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(54) Title: NANBV DIAGNOSTICS AND VACCINES

(57) Abstract

A family of cDNA sequences derived from hepatitis C virus (HCV) are provided. These sequences encode antigens which react immunologically with antibodies present in individuals with non-A non-B hepatitis (NANBH), but which generally are absent from individuals infected with hepatitis A virus (HAV) or hepatitis B virus (HBV), and also are absent from control individuals. A comparison of these cDNA sequences with the sequences in Genebank, and with the sequences of hepatitis delta virus (HDV) and HBV shows a lack of substantial homology. A comparison of the sequences of quences of hepatitis delta virus (HDV) and HBV shows a lack of substantial homology. A comparison of the sequences of quences of hepatitis delta virus (HDV) and HBV shows a lack of substantial homology. A comparison of the sequences of the HCV cDNA sequences are useful for the design of polynucleotide probes, and for the synthesis of polypeptides which may be useful for the diagnosis of HCV-induced NANBH, and for screening blood bank specimens and donors for HCV infection. In addition, these cDNA sequences may be useful for the synthesis of immunogenic polypeptides which may be used in vaccines for the treatment, prophylactic and/or therapeutic, of HCV infection. Polypeptides encoded within the cDNA sequences may also be used to raise antibodies against HCV antigenes, and for the purification of antibodies directed against HCV antigenes. These antibodies may be useful in immunoassays for detecting HCV antigenes associated with NANBH in individuals. The reagents provided in the invention also enable the isolation of NANBH agent(s), and the propagation of these agent(s) in tissue culture systems. Moreover, they provide reagents which are useful for screening for antiviral agents for HCV, particularly in tissue culture or animal model systems.

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NANBY DIAGNOSTICS AND VACCINES

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Technical Field

The invention relates to materials and methodologies for managing the spread of non-A, non-B
25 hepatitis virus (NANBV) infection. More specifically, it relates to diagnostic DNA fragments, diagnostic proteins, diagnostic antibodies and protective antigens and antibodies for an etiologic agent of NANB hepatitis, i.e., hepatitis C virus.

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U.S. Patent No. 4,491,632
U.S. Patent No. 4,493,890

15 Background Art

Non-A, Non-B hepatitis (NANBH) is a transmissible disease or family of diseases that are believed to be viral-induced, and that are distinguishable from other forms of viral-associated liver diseases,

- including that caused by the known hepatitis viruses, i.e., hepatitis A virus (HAV), hepatitis B virus (HBV), and delta hepatitis virus (HDV), as well as the hepatitis induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV). NANBH was first identified in transfused
- individuals. Transmission from man to chimpanzee and serial passage in chimpanzees provided evidence that NANBH is due to a transmissible infectious agent or agents. However, the transmissible agent responsible for NANBH is still unidentified and the number of agents which are causative of the disease are unknown.

Epidemiologic evidence is suggestive that there may be three types of NANBH: the water-borne epidemic type; the blood or needle associated type; and the sporadically occurring (community acquired) type.

However, the number of agents which may be the causative of NANBH are unknown.

Clinical diagnosis and identification of NANBH has been accomplished primarily by exclusion of other viral markers. Among the methods used to detect putative NANBV antigens and antibodies are agar-gel diffusion, counterimmunoelectrophoresis, immunofluorescence microscopy, immune electron microscopy, radioimmunoassay, and enzyme-linked immunosorbent assay. However, none of these assays has proved to be sufficiently sensitive, specific, and reproducible to be used as a diagnostic test for NANBH.

Until now there has been neither clarity nor agreement as to the identity or specificity of the antigen antibody systems associated with agents of NANBH. due, at least in part, to the prior or co-infection of HBV with NANBV in individuals, and to the known complexity of the soluble and particulate antigens associated with HBV, as well as to the integration of HBV DNA into the genome of liver cells. In addition, there is the possibility that NANBH is caused by more than one infectious agent, as well as the possibility that NANBH has been misdiagnosed. Moreover, it is unclear what the serological assays detect in the serum of patients with NANBH. It has been postulated that the agar-gel diffusion and counterimmunoelectrophoresis assays detect autoimmune responses or nonspecific protein interactions that sometimes occur between serum specimens, and that they do not represent specific NANBV antigen-antibody reactions. The immunofluorescence, and enzyme-linked immunosorbent, and radioimmunoassays 30 appear to detect low levels of a rheumatoid-factor-like material that is frequently present in the serum of patients with NANBH as well as in patients with other hepatic and nonhepatic diseases. Some of the reactivity

detected may represent antibody to host-determined cytoplasmic antigens.

There are a number of candidate NANBV. See, for example the reviews by Prince (1983), Feinstone and
Hoofnagle (1984), and Overby (1985, 1986, 1987) and the article by Iwarson (1987). However, there is no proof that any of these candidates represent the etiological agent of NANBH.

The demand for sensitive, specific methods for screening and identifying carriers of NANBV and NANBV contaminated blood or blood products is significant.

Post-transfusion hepatitis (PTH) occurs in approximately 10% of transfused patients, and NANBH accounts for up to 90% of these cases. The major problem in this disease is the frequent progression to chronic liver damage (25-55%).

Patient care as well as the prevention of transmission of NANBH by blood and blood products or by close personal contact require reliable diagnostic and prognostic tools to detect nucleic acids, antigens and antibodies related to NANBV. In addition, there is also a need for effective vaccines and immunotherapeutic therapeutic agents for the prevention and/or treatment of the disease.

25 Disclosure of the Invention

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The invention pertains to the isolation and characterization of a newly discovered etiologic agent of NANBH, hepatitis C virus (HCV). More specifically, the invention provides a family of cDNA replicas of portions of HCV genome. These cDNA replicas were isolated by a technique which included a novel step of screening expression products from cDNA libraries created from a particulate agent in infected tissue with sera from patients with NANBH to detect newly synthesized antigens derived from the genome of the heretofore unisolated and

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uncharacterized viral agent, and of selecting clones which produced products which reacted immunologically only with sera from infected individuals as compared to non-infected individuals.

Studies of the nature of the genome of the HCV, utilizing probes derived from the HCV cDNA, as well as sequence information contained within the HCV cDNA, are suggestive that HCV is a Flavivirus or a Flavi-like virus.

Portions of the cDNA sequences derived from HCV are useful as probes to diagnose the presence of virus in samples, and to isolate naturally occurring variants of the virus. These cDNAs also make available polypeptide sequences of HCV antigens encoded within the HCV genome(s) and permits the production of polypeptides which are use-15 ful as standards or reagents in diagnostic tests and/or as components of vaccines. Antibodies, both polyclonal and monoclonal, directed against HCV epitopes contained within these polypeptide sequences are also useful for diagnostic tests, as therapeutic agents, for screening of 20 antiviral agents, and for the isolation of the NANBV agent from which these cDNAs derive. In addition, by utilizing probes derived from these cDNAs it is possible to isolate and sequence other portions of the HCV genome, thus giving rise to additional probes and polypeptides which are 25 useful in the diagnosis and/or treatment, both prophylactic and therapeutic, of NANBH.

Accordingly with respect to polynucleotides, some aspects of the invention are: a purified HCV polynucleotide; a recombinant HCV polynucleotide; a 30 recombinant polynucleotide comprising a sequence derived from an HCV genome or from HCV cDNA; a recombinant polynucleotide encoding an epitope of HCV; a recombinant vector containing the any of the above recombinant polynucleotides, and a host cell transformed with any of these vectors.

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Other aspects of the invention are: a recombinant expression system comprising an open reading frame (ORF) of DNA derived from an HCV genome or from HCV cDNA, wherein the ORF is operably linked to a control sequence compatible with a desired host, a cell transformed with the recombinant expression system, and a polypeptide produced by the transformed cell.

Still other aspects of the invention are: purified HCV, a preparation of polypeptides from the 10 purified HCV; a purified HCV polypeptide; a purified polypeptide comprising an epitope which is immunologically identifiable with an epitope contained in HCV.

Included aspects of the invention are a recombinant HCV polypeptide; a recombinant polypeptide comprised of a sequence derived from an HCV genome or from HCV cDNA; a recombinant polypeptide comprised of an HCV epitope; and a fusion polypeptide comprised of an HCV polypeptide.

Also included in the invention are a monoclonal 20 antibody directed against an HCV epitope; and a purified preparation of polyclonal antibodies directed against an HCV epitope.

Another aspect of the invention is a particle which is immunogenic against HCV infection comprising a non-HCV polypeptide having an amino acid sequence capable of forming a particle when said sequence is produced in a eukaryotic host, and an HCV epitope.

Still another aspect of the invention is a polynucleotide probe for HCV.

Aspects of the invention which pertain to kits are those for: analyzing samples for the presence of polynucleotides derived from HCV comprising a polynucleotide probe containing a nucleotide sequence from HCV of about 8 or more nucleotides, in a suitable container; analyzing samples for the presence of an HCV 35

antigen comprising an antibody directed against the HCV antigen to be detected, in a suitable container; analyzing samples for the presence of an antibodies directed against an HCV antigen comprising a polypeptide containing an HCV epitope present in the HCV antigen, in a suitable container.

Other aspects of the invention are: a polypeptide comprised of an HCV epitope, attached to a solid substrate; and an antibody to an HCV epitope, attached to a solid substrate.

Still other aspects of the invention are: a method for producing a polypeptide containing an HCV epitope comprising incubating host cells transformed with an expression vector containing a sequence encoding a polypeptide containing an HCV epitope under conditions which allow expression of said polypeptide; and a polypeptide containing an HCV epitope produced by this method.

The invention also includes a method for

20 detecting HCV nucleic acids in a sample comprising
reacting nucleic acids of the sample with a probe for an
HCV polynucleotide under conditions which allow the
formation of a polynucleotide duplex between the probe and
the HCV nucleic acid from the sample; and detecting a

25 polynucleotide duplex which contains the probe.

Immunoassays are also included in the invention.

These include an immunoassay for detecting an HCV antigen comprising incubating a sample suspected of containing an HCV antigen with a probe antibody directed against the HCV antigen to be detected under conditions which allow the formation of an antigen-antibody complex; and detecting an antigen-antibody complex containing the probe antibody. An immunoassay for detecting antibodies directed against an HCV antigen comprising incubating a sample suspected of containing anti-HCV antibodies with a probe polypeptide

which contains an epitope of the HCV, under conditions which allow the formation of an antibody-antigen complex; and detecting the antibody-antigen complex containing the probe antigen.

Also included in the invention are vaccines for treatment of HCV infection comprising an immunogenic peptide containing an HCV epitope, or an inactivated preparation of HCV, or an attenuated preparation of HCV.

Another aspect of the invention is a tissue culture grown cell infected with HCV.

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Yet another aspect of the invention is a method for producing antibodies to HCV comprising administering to an individual an isolated immunogenic polypeptide containing an HCV epitope in an amount sufficient to produce an immune response.

Still another aspect of the invention is a method for isolating cDNA derived from the genome of an unidentified infectious agent, comprising: (a) providing host cells transformed with expression vectors containing a cDNA library prepared from nucleic acids isolated from tissue infected with the agent and growing said host cells under conditions which allow expression of polypeptide(s) encoded in the cDNA; (b) interacting the expression products of the cDNA with an antibody containing body component of an individual infected with said infectious agent under conditions which allow an immunoreaction, and detecting antibody-antigen complexes formed as a result of the interacting; (c) growing host cells which express polypeptides that form antibody-antigen complexes in step (b) under conditions which allow their growth as individual clones and isolating said clones; (d) growing cells from the clones of (c) under conditions which allow expression of polypeptide(s) encoded within the cDNA, and interacting the expression products with antibody containing body components of individuals other than the

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individual in step (a) who are infected with the infectious agent and with control individuals uninfected with the agent, and detecting antibody-antigen complexes formed as a result of the interacting; (e) growing host 5 cells which express polypeptides that form antibodyantigen complexes with antibody containing body components of infected individuals and individuals suspected of being infected, and not with said components of control individuals, under conditions which allow their growth as individual clones and isolating said clones; and (f) isolating the cDNA from the host cell clones of (e).

Brief Description of the Drawings

Fig. 1 shows the double-stranded nucleotide sequence of the HCV cDNA insert in clone 5-1-1, and the putative amino acid sequence of the polypeptide encoded therein.

Fig. 2 shows the homologies of the overlapping HCV cDNA sequences in clones 5-1-1, 81, 1-2, and 91.

Fig. 3 shows a composite sequence of HCV cDNA derived from overlapping clones 81, 1-2, and 91, and the amino acid sequence encoded therein.

Fig. 4 shows the double-stranded nucleotide sequence of the HCV cDNA insert in clone 81, and the putative amino acid sequence of the polypeptide encoded therein.

Fig. 5 shows the HCV cDNA sequence in clone 36, the segment which overlaps the NANBV cDNA of clone 81, and the polypeptide sequence encoded within clone 36.

Fig. 6 shows the combined ORF of HCV cDNAs in clones 36 and 81, and the polypeptide encoded therein.

Fig. 7 shows the HCV cDNA sequence in clone 32, the segment which overlaps clone 81, and the polypeptide encoded therein.

Fig. 8 shows the HCV cDNA sequence in clone 35, the segment which overlaps clone 36, and the polypeptide encoded therein.

Fig. 9 shows the combined ORF of HCV cDNAs in clones 35, 36, 81, and 32, and the polypeptide encoded therein.

Fig. 10 shows the HCV cDNA sequence in clone 37b, the segment which overlaps clone 35, and the polypeptide encoded therein.

Fig. 11 shows the HCV cDNA sequence in clone 33b, the segment which overlaps clone 32, and the polypeptide encoded therein.

Fig. 12 shows the HCV cDNA sequence in clone 40b, the segment which overlaps clone 37b, and the polypeptide encoded therein.

Fig. 13 shows the HCV cDNA sequence in clone 25c, the segment which overlaps clone 33b, and the polypeptide encoded therein.

Fig. 14 shows the nucleotide sequence and polypeptide encoded therein of the ORF which extends through the HCV cDNAs in clones 40b, 37b, 35, 36, 81, 32, 33b, and 25c.

Fig. 15 shows the HCV cDNA sequence in clone 33c, the segment which overlaps clones 40b and 33c, and the amino acids encoded therein.

Fig. 16 shows the HCV cDNA sequence in clone 8h, the segment which overlaps clone 33c, and the amino acids encoded therein.

Fig. 17 shows the HCV cDNA sequence in clone 7e, 30 the segment which overlaps clone 8h, and the amino acids encoded therein.

Fig. 18 shows the HCV cDNA sequence in clone 14c, the segment which overlaps clone 25c, and the amino acids encoded therein.

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Fig. 19 shows the HCV cDNA sequence in clone 8f, the segment which overlaps clone 14c, and the amino acids encoded therein.

Fig. 20 shows the HCV cDNA sequence in clone 33f, the segment which overlaps clone 8f, and the amino acids encoded therein.

Fig. 21 shows the HCV cDNA sequence in clone 33g, the segment which overlaps clone 33f, and the amino acids encoded therein.

Fig. 22 shows the HCV cDNA sequence in clone 7f, the segment which overlaps the sequence in clone 7e, and the amino acids encoded therein.

Fig. 23 shows the HCV cDNA sequence in clone 11b, the segment which overlaps the sequence in clone 7f, and the amino acids encoded therein.

Fig. 24 shows the HCV cDNA sequence in clone 14i, the segment which overlaps the sequence in clone 11b, and the amino acids encoded therein.

Fig. 25 shows the HCV cDNA sequence in clone 39c, the segment which overlaps the sequence in clone 33g, and the amino acids encoded therein.

Fig. 26 shows a composite HCV cDNA sequence derived from the aligned cDNAs in clones 14i, 11b, 7f, 7e, 8h, 33c 40b 37b 35 36, 81, 32, 33b, 25c, 14c, 8f, 33f, and 33g; also shown is the amino acid sequence of the polypeptide encoded in the extended ORF in the derived sequence.

Fig. 27 shows the sequence of the HCV cDNA in clone 12f, the segment which overlaps clone 14i, and the amino acids encoded therein.

Fig. 28 shows the sequence of the HCV cDNA in clone 35f, the segment which overlaps clone 39c, and the amino acids encoded therein.

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Fig. 29 shows the sequence of the HCV cDNA in clone 19g, the segment which overlaps clone 35f, and the amino acids encoded therein.

Fig. 30 shows the sequence of clone 26g, the segment which overlaps clone 19g, and the amino acids encoded therein.

Fig. 31 shows the sequence of clone 15e, the segment which overlaps clone 26g, and the amino acids encoded therein.

Fig. 32 shows the sequence in a composite cDNA, which was derived by aligning clones 12f through 15e in the 5' to 3' direction; it also shows the amino acids encoded in the continuous ORF.

Fig. 33 shows a photograph of Western blots of a fusion protein, $SOD-NANB_{5-1-1}$, with chimpanzee serum from chimpanzees infected with BB-NANB, HAV, and HBV.

Fig. 34 shows a photograph of Western blots of a fusion protein, SOD-NANB $_{5-1-1}$, with serum from humans infected with NANBV, HAV, HBV, and from control humans.

Fig. 35 is a map showing the significant features of the vector pAB24.

Fig. 36 shows the putative amino acid sequence of the carboxy-terminus of the fusion polypeptide C100-3 and the nucleotide sequence encoding it.

Fig. 37A is a photograph of a coomassie blue stained polyacrylamide gel which identifies C100-3 expressed in yeast.

Fig. 37B shows a Western blot of C100-3 with serum from a NANBV infected human.

Fig. 38 shows an autoradiograph of a Northern blot of RNA isolated from the liver of a BB-NANBV infected chimpanzee, probed with BB-NANBV cDNA of clone 81.

Fig. 39 shows an autoradiograph of NANBV nucleic acid treated with RNase A or DNase I, and probed with BB-NANBV cDNA of clone 81.

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Fig. 40 shows an autoradiograph of nucleic acids extracted from NANBV particles captured from infected plasma with anti-NANB $_{5-1-1}$, and probed with 32 P-labeled NANBV cDNA from clone 81.

Fig. 41 shows autoradiographs of filters containing isolated NANBV nucleic acids, probed with ³²P-labeled plus and minus strand DNA probes derived from NANBV cDNA in clone 81.

Fig. 42 shows the homologies between a polypeptide encoded in HCV cDNA and an NS protein from Dengue flavivirus.

Fig. 43 shows a histogram of the distribution of HCV infection in random samples, as determined by an ELISA screening.

Fig. 44 shows a histogram of the distribution of HCV infection in random samples using two configurations of immunoglobulin-enzyme conjugate in an ELISA assay.

Fig. 45 shows the sequences in a primer mix, derived from a conserved sequence in NS1 of flaviviruses.

Fig. 46 shows the HCV cDNA sequence in clone k9-1, the segment which overlaps the cDNA in Fig. 26, and the amino acids encoded therein.

Fig. 47 shows the sequence in a composite cDNA which was derived by aligning clones k9-1 through 15e in the 5' to 3' direction; it also shows the amino acids encoded in the continuous ORF.

Modes for Carrying Out the Invention

30 I. Definitions

The term "hepatitis C virus" has been reserved by workers in the field for an heretofore unknown etiologic agent of NANBH. Accordingly, as used herein, "hepatitis C virus" (HCV) refers to an agent causitive of NANBH, which was formerly referred to as NANBV and/or BB-

NANBV. The terms HCV, NANBV, and BB-NANBV are used interchangeably herein. As an extension of this terminology, the disease caused by HCV, formerly called NANB hepatitis (NANBH), is called hepatitis C. The terms NANBH and hepatitis C may be used interchangeably herein.

The term "HCV", as used herein, denotes a viral species which causes NANBH, and attenuated strains or defective interfering particles derived therefrom. As shown infra., the HCV genome is comprised of RNA. It is known that RNA containing viruses have relatively high rates of spontaneous mutation, i.e., reportedly on the order of 10⁻³ to 10⁻⁴ per nucleotide (Fields & Knipe (1986)). Therefore, there are multiple strains within the HCV species described infra. The compositions and methods described herein, enable the propagation, identification, detection, and isolation of the various related strains. Moreover, they also allow the preparation of diagnostics and vaccines for the various strains, and have utility in screening procedures for anti-viral agents for

The information provided herein, although derived from one strain of HCV, hereinafter referred to as CDC/HCV1, is sufficient to allow a viral taxonomist to identify other strains which fall within the species. As described herein, we have discovered that HCV is a Flavivirus or Flavi-like virus. The morphology and composition of Flavivirus particles are known, and are discussed in Brinton (1986). Generally, with respect to morphology, Flaviviruses contain a central nucleocapsid surrounded by a lipid bilayer. Virions are spherical and have a diameter of about 40-50 nm. Their cores are about 25-30 nm in diameter. Along the outer surface of the virion envelope are projections that are about 5-10 nm long with terminal knobs about 2 nm in diameter.

HCV encodes an epitope which is immunologically identifiable with an epitope in the HCV genome from which the cDNAs described herein are derived; preferably the epitope is encoded in a cDNA described herein. 5 epitope is unique to HCV when compared to other known Flaviviruses. The uniqueness of the epitope may be determined by its immunological reactivity with HCV and lack of immunological reactivity with other Flavivirus species. Methods for determining immunological reactivity 10 are known in the art, for example, by radioimmunoassay, by Elisa assay, by hemagglutination, and several examples of suitable techniques for assays are provided herein.

In addition to the above, the following parameters are applicable, either alone or in combination, in identifying a strain as HCV. Since HCV strains are evolutionarily related, it is expected that the overall homology of the genomes at the nucleotide level will be about 40% or greater, preferably about 60% or greater, and even more preferably about 80% or greater; and in addition 20 that there will be corresponding contiguous sequences of at least about 13 nucleotides. The correspondence between the putative HCV strain genomic sequence and the CDC/CH1 HCV cDNA sequence can be determined by techniques known in the art. For example, they can be determined by a direct 25 comparison of the sequence information of the polynucleotide from the putative HCV, and the HCV cDNA sequence(s) described herein. For example, also, they can be determined by hybridization of the polynucleotides under conditions which form stable duplexes between 30 homologous regions (for example, those which would be used prior to S, digestion), followed by digestion with single stranded specific nuclease(s), followed by size determination of the digested fragments.

Because of the evolutionary relationship of the strains of HCV, putative HCV strains are identifiable by

their homology at the polypeptide level. Generally, HCV strains are more than about 40% homologous, preferably more than about 60% homologous, and even more preferably more than about 80% homologous at the polypeptide level.

The techniques for determining amino acid sequence homology are known in the art. For example, the amino acid sequence may be determined directly and compared to the sequences provided herein. For example also, the nucleotide sequence of the genomic material of the putative HCV may be determined (usually via a cDNA

intermediate); the amino acid sequence encoded therein can be determined, and the corresponding regions compared.

As used herein, a polynucleotide "derived from" a designated sequence, for example, the HCV cDNA,

particularly those exemplified in Figs. 1-32, or from an HCV genome, refers to a polynucleotide sequence which is comprised of a sequence of approximately at least about 6 nucleotides, is preferably at least about 8 nucleotides, is more preferably at least about 10-12 nucleotides, and

even more preferably at least about 15-20 nucleotides corresponding, i.e., homologous to or complementary to, a region of the designated nucleotide sequence. Preferably, the sequence of the region from which the polynucleotide is derived is homologous to or complementary to a sequence

which is unique to an HCV genome. Whether or not a sequence is unique to the HCV genome can be determined by techniques known to those of skill in the art. For example, the sequence can be compared to sequences in databanks, e.g., Genebank, to determine whether it is present in the uninfected host or other organisms. The

present in the uninfected host or other organisms. The sequence can also be compared to the known sequences of other viral agents, including those which are known to induce hepatitis, e.g., HAV, HBV, and HDV, and to other members of the Flaviviridae. The correspondence or non-

35 correspondence of the derived sequence to other sequences

can also be determined by hybridization under the appropriate stringency conditions. Hybridization techniques for determining the complementarity of nucleic acid sequences are known in the art, and are discussed infra. 5 See also, for example, Maniatis et al. (1982). In addition, mismatches of duplex polynucleotides formed by hybridization can be determined by known techniques, including for example, digestion with a nuclease such as S1 that specifically digests single-stranded areas in duplex polynucleotides. Regions from which typical DNA sequences may be "derived" include but are not limited to, for example, regions encoding specific epitopes, as well as non-transcribed and/or non-translated regions.

The derived polynucleotide is not necessarily 15 physically derived from the nucleotide sequence shown, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence of bases in the 20 region(s) from which the polynucleotide is derived. addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use.

Similarly, a polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence, for 25 example, the sequences in Figs. 1-32, or from an HCV genome, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion 30 consists of at least 3-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not 35 necessarily translated from a designated nucleic acid

sequence, for example, the sequences in Figs. 1-26, or from an HCV genome; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system, or isolation from mutated HCV.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature or in the form of a library; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature.

The term "polynucleotide" as used herein refers 15 to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, as well as double- and single stranded RNA. It also includes modified, for example, by methylation and/or by capping, 20 and unmodified forms of the polynucleotide.

As used herein, the term "HCV containing a sequence corresponding to a cDNA" means that the HCV contains a polynucleotide sequence which is homologous to 25 or complementary to a sequence in the designated DNA; the degree of homology or complementarity to the cDNA will be approximately 50% or greater, will preferably be at least about 70%, and even more preferably will be at least about 90%. The sequences which correspond will be at least about 70 nucleotides, preferably at least about 80 nucleotides, and even more preferably at least about 90 nucleotides in length. The correspondence between the HCV sequence and the cDNA can be determined by techniques known in the art, including, for example, a direct comparison of the sequenced material with the cDNAs

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described, or hybridization and digestion with single strand nucleases, followed by size determination of the digested fragments.

The term "purified of viral polynucleotide" 5 refers to an HCV genome or fragment thereof which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and even more preferably less than about 90% of polypeptides with which the viral polynucleotide is naturally associated. Techniques for purifying viral polynucleotides from viral particles are known in the art, and include for example, disruption of the particle with a chaotropic agent, and separation of the polynucleotide(s) and polypeptides by ion-exchange chromatography, affinity chromatography, and sedimentation according to density.

The term "purified viral polypeptided" refers to an HCV polypeptide or fragment thereof which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and even more preferably less than about 90%, of cellular components with which the viral polypeptide is naturally associated. Techniques for purifying viral polypeptides are known in the art, and examples of these techniques are discussed infra.

"Recombinant host cells", "host cells", "cells", 25 "cell lines", "cell cultures:, and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transfected. 30 understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent

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sequences.

to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

A "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding 5 sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'terminus. A coding sequence can include, but is not limited to mRNA, cDNA, and recombinant polynucleotide sequences.

"Immunologically identifiable with/as" refers to the presence of epitope(s) and polypeptides(s) which are also present in and are unique to the designated polypeptide(s), usually HCV proteins. Immunological identity may be determined by antibody binding and/or 15 competition in binding; these techniques are known to those of average skill in the art, and are also illustrated infra. The uniqueness of an epitope can also be determined by computer searches of known data banks, e.g. Genebank, for the polynucleotide sequences which encode 20 the epitope, and by amino acid sequence comparisons with other known proteins.

As used herein, "epitope" refers to an antigenic determinant of a polypeptide; an epitope could comprise 3 amino acids in a spatial conformation which is unique to 25 the epitope, generally an epitope consists of at least 5 such amino acids, and more usually, consists of at least 8-10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2-30 dimensional nuclear magnetic resonance.

A polypeptide is "immunologically reactive" with an antibody when it binds to an antibody due to antibody recognition of a specific epitope contained within the 5 polypeptide. Immunological reactivity may be determined 35 by antibody binding, more particularly by the kinetics of

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antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an epitope against which the antibody is directed. techniques for determining whether a polypeptide is im-5 munologically reactive with an antibody are known in the art.

As used herein, the term "immunogenic polypeptide containing an HCV epitope" includes naturally occurring HCV polypeptides or fragments thereof, as well as polypeptides prepared by other means, for example, chemical synthesis, or the expression of the polypeptide in a recombinant organism.

The term "polypeptide" refers to a molecular chain of amino acids and does not refer to a specific 15 length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of This term also does not refer to postpolypeptide. expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, or f-mating. 25 exogenous polynucleotide may be maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. "Treatment" as used herein refers to prophylaxis and/or therapy.

An "individual", as used herein, refers to vertebrates, particularly members of the mammalian species, and includes but is not limited to domestic animals, sports animals, primates, and humans.

As used herein, the "plus strand" of a nucleic acid contains the sequence that encodes the polypeptide. 35

The "minus strand" contains a sequence which is complementary to that of the "plus strand".

As used herein, a "positive stranded genome" of a virus is one in which the genome, whether RNA or DNA, is single-stranded and which encodes a viral polypeptide(s). Examples of positive stranded RNA viruses include Togaviridae, Coronaviridae, Retroviridae, Picornaviridae, and Caliciviridae. Included also, are the Flaviviridae, which were formerly classified as Togaviradae. See Fields & Knipe (1986).

As used herein, "antibody containing body component" refers to a component of an individual's body which is a source of the antibodies of interest. Antibody containing body components are known in the art, and include but are not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, white blood cells, and myelomas.

As used herein, "purified HCV" refers to a
preparation of HCV which has been isolated from the
cellular constituents with which the virus is normally
associated, and from other types of viruses which may be
present in the infected tissue. The techniques for
isolating viruses are known to those of skill in the art,
and include, for example, centrifugation and affinity
chromatography; a method of preparing purified HCV is
discussed infra.

II. Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Maniatis, Fitsch & Sambrook,

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MOLECULAR CLONING; A LABORATORY MANUAL (1982); DNA CLON-ING, VOLUMES I AND II (D.N Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IM-MUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds 1986).

All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby incorporated herein by reference.

The useful materials and processes of the present invention are made possible by the provision of a family of closely homologous nucleotide sequences isolated from a cDNA library derived from nucleic acid sequences present in the plasma of an HCV infected chimpanzee. This family of nucleotide sequences is not of human or chimpanzee origin, since it hybridizes to neither human nor chimpanzee genomic DNA from uninfected individuals, since nucleotides of this family of sequences are present only in liver and plasma of chimpanzees with HCV infection, and since the sequence is not present in Genebank. In addition, the family of sequences shows no significant homology to sequences contained within the HBV genome.

The sequence of one member of the family, contained within clone 5-1-1, has one continuous open reading frame (ORF) which encodes a polypeptide of approximately 50 amino acids. Sera from HCV infected humans 5 contain antibodies which bind to this polypeptide, whereas sera from non-infected humans do not contain antibodies to this polypeptide. Finally, whereas the sera from uninfected chimpanzees do not contain antibodies to this polypeptide, the antibodies are induced in chimpanzees 10 following acute NANBH infection. Moreover, antibodies to this polypeptide are not detected in chimps and humans infected with HAV and HBV. By these criteria the sequence is a cDNA to a viral sequence, wherein the virus causes or is associated with NANBH; this cDNA sequence is shown in 15 Fig. 1. As discussed infra, the cDNA sequence in clone 5-1-1 differs from that of the other isolated cDNAs in that it contains 28 extra base pairs.

A composite of other identified members of the cDNA family, which were isolated using as a probe a 20 synthetic sequence equivalent to a fragment of the cDNA in clone 5-1-1, is shown in Fig. 3. A member of the cDNA family which was isolated using a synthetic sequence derived from the cDNA in clone 81 is shown in Fig. 5, and the composite of this sequence with that of clone 81 is 25 shown in Fig. 6. Other members of the cDNA family, including those present in clones 12f, 14i, 11b, 7f, 7e, 8h, 33c, 40b, 37b, 35, 36, 81, 32, 33b, 25c, 14c, 8f, 33f, and 33g, 39c, 35f, 19g, 26g and 15e are described in Section IV.A.. A composite of the cDNAs in these clones 30 is described in Section IV.A.19, and shown in Fig. 32. The composite cDNA shows that it contains one continuous ORF, and thus encodes a polyprotein. This data is consistent with the suggestion, discussed infra., that HCV is a flavivirus or flavi-like virus.

The availability of this family of cDNAs shown in Figs. 1-32, inclusive, permits the construction of DNA probes and polypeptides useful in diagnosing NANBH due to HCV infection and in screening blood donors as well as 5 donated blood and blood products for infection. example, from the sequences it is possible to synthesize DNA oligomers of about 8-10 nucleotides, or larger, which are useful as hybridization probes to detect the presence of the viral genome in, for example, sera of subjects suspected of harboring the virus, or for screening donated blood for the presence of the virus. The family of cDNA sequences also allows the design and production of HCV specific polypeptides which are useful as diagnostic reagents for the presence of antibodies raised during NANBH. Antibodies to purified polypeptides derived from the cDNAs may also be used to detect viral antigens in infected individuals and in blood.

Knowledge of these cDNA sequences also enable the design and production of polypeptides which may be used as vaccines against HCV and also for the production of antibodies, which in turn may be used for protection against the disease, and/or for therapy of HCV infected individuals.

further characterization of the HCV genome.

Polynucleotide probes derived from these sequences may be used to screen cDNA libraries for additional overlapping cDNA sequences, which, in turn, may be used to obtain more overlapping sequences. Unless the genome is segmented and the segments lack common sequences, this technique may be used to gain the sequence of the entire genome. However, if the genome is segmented, other segments of the genome can be obtained by repeating the lambda-gtl1 serological screening procedure used to isolate the cDNA clones

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described herein, or alternatively by isolating the genome from purified HCV particles.

The family of cDNA sequences and the polypeptides derived from these sequences, as well as antibodies directed against these polypeptides are also useful in the isolation and identification of the BB-NANBV agent(s). For example, antibodies directed against HCV epitopes contained in polypeptides derived from the cDNAs may be used in processes based upon affinity chromatography to isolate the virus. Alternatively, the antibodies may be used to identify viral particles isolated by other techniques. The viral antigens and the genomic material within the isolated viral particles may then be further characterized.

The information obtained from further sequencing of the HCV genome(s), as well as from further characterization of the HCV antigens and characterization of the genome enables the design and synthesis of additional probes and polypeptides and antibodies which may be used for diagnosis, for prevention, and for therapy of HCV induced NANBH, and for screening for infected blood and blood-related products.

The availability of probes for HCV, including antigens and antibodies, and polynucleotides derived from the genome from which the family of cDNAs is derived also allows for the development of tissue culture systems which will be of major use in elucidating the biology of HCV. This in turn, may lead to the development of new treatment regimens based upon antiviral compounds which 30 preferentially inhibit the replication of, or infection by

The method used to identify and isolate the etiologic agent for NANBH is novel, and it may be applicable to the identification and/or isolation of hereto-35 fore uncharacterized agents which contain a genome, and

which are associated with a variety of diseases, including those induced by viruses, viroids, bacteria, fungi and parasites. In this method, a cDNA library was created from the nucleic acids present in infected tissue from an infected individual. The library was created in a vector which allowed the expression of polypeptides encoded in the cDNA. Clones of host cells containing the vector, which expressed an immunologically reactive fragment of a polypeptide of the etiologic agent, were selected by immunological screening of the expression products of the 10 library with an antibody containing body component from another individual previously infected with the putative agent. The steps in the immunological screening technique included interacting the expression products of the cDNA containing vectors with the antibody containing body 15 component of a second infected individual, and detecting the formation of antibody-antigen complexes between the expression product(s) and antibodies of the second infected individual. The isolated clones are screened further immunologically by interacting their expression 20 products with the antibody containing body components of other individuals infected with the putative agent and with control individuals uninfected with the putative agent, and detecting the formation of antigen-antibody complexes with antibodies from the infected individuals; 25 and the cDNA containing vectors which encode polypeptides which react immunologically with antibodies from infected individuals and individuals suspected of being infected with the agent, but not with control individuals are isolated. The infected individuals used for the construc-30 tion of the cDNA library, and for the immunological screening need not be of the same species.

The cDNAs isolated as a result of this method, and their expression products, and antibodies directed; against the expression products, are useful in character-

izing and/or capturing the etiologic agent. As described in more detail infra, this method has been used successfully to isolate a family of cDNAs derived from the HCV genome.

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II.A. Preparation of the cDNA Sequence

Pooled serum from a chimpanzee with chronic HCV infection and containing a high titer of the virus, i.e., at least 10⁵ chimp infectious doses/ml (CID/ml) was used 10 to isolate viral particles; nucleic acids isolated from these particles was used as the template in the construction of a cDNA library to the viral genome. procedures for isolation of putative HCV particles and for constructing the cDNA library in lambda-gtll is discussed in Section IV.A.1. Lambda-gtll is a vector that has been developed specifically to express inserted cDNAs as fusion polypeptides with beta-galactosidase and to screen large numbers of recombinant phage with specific antisera raised against a defined antigen. The lambda-gtl1 cDNA library 20 generated from a cDNA pool containing cDNA of approximate mean size of 200 base pairs was screened for encoded epitopes that could bind specifically with sera derived from patients who had previously experienced NANB hepatitis. Huynh, T.V. et al. (1985). Approximately 10⁶ 25 phages were screened, and five positive phages were identified, purified, and then tested for specificity of binding to sera from different humans and chimpanzees previously infected with the HCV agent. One of the phages, 5-1-1, bound 5 of the 8 human sera tested. This 30 binding appeared selective for sera derived from patients with prior NANB hepatitis infections since 7 normal blood donor sera did not exhibit such binding.

The sequence of the cDNA in recombinant phage 5-I-1 was determined, and is shown in Fig. 1. The 35 polypeptide encoded by this cloned cDNA, which is in the same translational frame as the N-terminal beta-Galactosidase moiety of the fusion polypeptide is shown above the nuclectide sequence. This translational ORF, therefore, encodes an epitope(s) specifically recognized by sera from patients with NANB hepatitis infections.

The availability of the cDNA in recombinant phage 5-1-1 has allowed for the isolation of other clones containing additional segments and/or alternative segments of cDNA to the viral genome. The lambda-gtll cDNA library described supra, was screened using a synthetic polynucleotide derived from the sequence of the cloned 5-1-1 cDNA. This screening yielded three other clones, which were identified as 81, 1-2 and 91; the cDNAs contained within these clones were sequenced. See Sections IV.A.3. and IV.A.4. The homologies between the four independent clones are shown in Fig. 2, where the homologies are indicated by the vertical lines. Sequences of nucleotides present uniquely in clones 5-1-1, 81, and 91 are indicated by small letters.

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The cloned cDNAs present in recombinant phages in clones 5-1-1, 81, 1-2, and 91 are highly homologous, and differ in only two regions. First, nucleotide number 67 in clone 1-2 is a thymidine, whereas the other three clones contain a cytidine residue in this position. This substitution, however, does not alter the nature of the encoded amino acid.

The second difference between the clones is that clone 5-1-1 contains 28 base pairs at its 5'-terminus which are not present in the other clones. The extra sequence may be a 5'-terminal cloning artifact; 5'-terminal cloning artifacts are commonly observed in the products of cDNA methods.

Synthetic sequences derived from the 5'-region and the 3'-region of the HCV cDNA in clone 81 were used to screen and isolate cDNAs from the lambda-gtll NANBV cDNA

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library, which overlapped clone 81 cDNA (Section IV.A.5.). The sequences of the resulting cDNAs, which are in clone 36 and clone 32, respectively, are shown in Fig. 5 and Fig. 7.

Similarly, a synthetic polynucleotide based on the 5'-region of clone 36 was used to screen and isolate cDNAs from the lambda gt-11 NANBV cDNA library which overlapped clone 36 cDNA (Section IV.A.8.). A purified clone of recombinant phage-containing cDNA which hybridized to the synthetic polynucleotide probe was named clone 35 and the NANBV cDNA sequence contained within this clone is shown in Fig. 8.

By utilizing the technique of isolating overlapping cDNA sequences, clones containing additional upstream and downstream HCV cDNA sequences have been obtained. The isolation of these clones, is described infra in Section IV.A.

Analysis of the nucleotide sequences of the HCV cDNAs encoded within the isolated clones show that the composite cDNA contains one long continuous ORF. Fig. 26 shows the sequence of the composite cDNA from these clones, along with the putative HCV polypeptide encoded therein.

The description of the method to retrieve the CDNA sequences is mostly of historical interest. The resultant sequences (and their complements) are provided herein, and the sequences, or any portion thereof, could be prepared using synthetic methods, or by a combination of synthetic methods with retrieval of partial sequences using methods similar to those described herein.

Lambda-gtl1 strains replicated from the HCV cDNA library and from clones 5-1-1, 81, 1-2 and 91 have been deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn

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Dr., Rockville, Maryland 20852, and have been assigned the following Accession Numbers.

lambda-gtl <u>l</u>	ATCC No.	Deposit Date
HCV cDNA library	40394	1 Dec. 1987
clone 81	40388	17 Nov. 1987
clone 91	40389	17 Nov. 1987
clone 1-2	40390	17 Nov. 1987
clone 5-1-1	40391	18 Nov. 1987

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Upon allowance and issuance of this application as a United States Patent, all restriction on availability of these deposits will be irrevocably removed; and access to the designated deposits will be available during pendency of the above-named application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 1.22. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. These deposits and other deposited materials mentioned herein are intended for convenience only, and are not required to practice the present invention in view of the description here. The HCV cDNA sequences in all of the deposited materials are incorporated herein by reference.

The description above, of "walking" the genome by isolating overlapping cDNA sequences from the HCV lambda gt-11 library provides one method by which cDNAs corresponding to the entire HCV genome may be isolated. However, given the information provided herein, other methods for isolating these cDNAs are obvious to one of skill in the art. Some of these methods are described in Section IV.A., infra.

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II.B. Preparation of Viral Polypeptides and Fragments

The availability of cDNA sequences, either those isolated by utilizing the cDNA sequences in Figs. 1-32, as discussed infra, as well as the cDNA sequences in these 5 figures, permits the construction of expression vectors encoding antigenically active regions of the polypeptide encoded in either strand. These antigenically active regions may be derived from coat or envelope antigens or from core antigens, including, for example, polynucleotide 10 binding proteins, polynucleotide polymerase(s), and other viral proteins required for the replication and/or assembly of the virus particle. Fragments encoding the desired polypeptides are derived from the cDNA clones using conventional restriction digestion or by synthetic 15 methods, and are ligated into vectors which may, for example, contain portions of fusion sequences such as beta-Galactosidase or superoxide dismutase (SOD), preferably SOD. Methods and vectors which are useful for the production of polypeptides which contain fusion sequences 20 of SOD are described in European Patent Office Publication number 0196056, published October 1, 1986. Vectors encoding fusion polypeptides of SOD and HCV polypeptides, i.e., $NANB_{5-1-1}$, $NANB_{81}$, and C100-3, which is encoded in a composite of HCV cDNAs, are described in Sections IV.B.1, 25 IV.B.2, and IV.B.4, respectively. Any desired portion of the HCV cDNA containing an open reading frame, in either sense strand, can be obtained as a recombinant polypeptide, such as a mature or fusion protein; alternatively, a polypeptide encoded in the cDNA can be 30 provided by chemical synthesis.

The DNA encoding the desired polypeptide, whether in fused or mature form, and whether or not containing a signal sequence to permit secretion, may be ligated into expression vectors suitable for any convenient host. Both eukaryotic and prokaryotic host

systems are presently used in forming recombinant polypeptides, and a summary of some of the more common control systems and host cell lines is given in Section III.A., infra. The polypeptide is then isolated from 5 lysed cells or from the culture medium and purified to the extent needed for its intended use. Purification may be by techniques known in the art, for example, salt fractionation, chromatography on ion exchange resins, affinity chromatography, centrifugation, and the like. 10 for example, Methods in Enzymology for a variety of methods for purifying proteins. Such polypeptides can be used as diagnostics, or those which give rise to neutralizing antibodies may be formulated into vaccines. Antibodies raised against these polypeptides can also be used 15 as diagnostics, or for passive immunotherapy. In addition, as discussed in Section II.J. herein below, antibodies to these polypeptides are useful for isolating and identifying HCV particles.

The HCV antigens may also be isolated from HCV virions. The virions may be grown in HCV infected cells in tissue culture, or in an infected host.

II.C. <u>Preparation of Antigenic Polypeptides and Conjugation with Carrier</u>

25 An antigenic region of a polypeptide is generally relatively small—typically 8 to 10 amino acids or less in length. Fragments of as few as 5 amino acids may characterize an antigenic region. These segments may correspond to regions of HCV antigen. Accordingly, using the cDNAs of HCV as a basis, DNAs encoding short segments of HCV polypeptides can be expressed recombinantly either as fusion proteins, or as isolated polypeptides. In addition, short amino acid sequences can be conveniently obtained by chemical synthesis. In instances wherein the synthesized polypeptide is correctly configured so as to

provide the correct epitope, but is too small to be immunogenic, the polypeptide may be linked to a suitable carrier.

A number of techniques for obtaining such link-5 age are known in the art, including the formation of disulfide linkages using N-succinimidy1-3-(2-pyridy1thio)propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) obtained from Pierce Company, Rockford, Illinois, (if the peptide lacks 10 a sulfhydryl group, this can be provided by addition of a cysteine residue.) These reagents create a disulfide linkage between themselves and peptide cysteine residues on one protein and an amide linkage through the epsilonamino on a lysine, or other free amino group in the other. 15 A variety of such disulfide/amide-forming agents are known. See, for example, Immun. Rev. (1982) 62:185. Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thio-etherforming agents are commercially available and include re-20 active esters of 6-maleimidocaproic acid, 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid, and the like. carboxyl groups can be activated by combining them with succinimide or 1-hydroxy1-2-nitro-4-sulfonic acid, sodium 25 salt. The foregoing list is not meant to be exhaustive, and modifications of the named compounds can clearly be used.

Any carrier may be used which does not itself induce the production of antibodies harmful to the host.

30 Suitable carriers are typically large, slowly metabolized macromolecules such as proteins; polysaccharides, such as latex functionalized sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids, such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles, see, for

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example, section II.D. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those skilled in the art.

II.D. Preparation of Hybrid Particle Immunogens Containing HCV Epitopes

The immunogenicity of the epitopes of HCV may also be enhanced by preparing them in mammalian or yeast 10 systems fused with or assembled with particle-forming proteins such as, for example, that associated with hepatitis B surface antigen. Constructs wherein the NANBV epitope is linked directly to the particle-forming protein coding sequences produce hybrids which are immunogenic 15 with respect to the HCV epitope. In addition, all of the vectors prepared include epitopes specific to HBV, having various degrees of immunogenicity, such as, for example, the pre-S peptide. Thus, particles constructed from particle forming protein which include HCV sequences are immunogenic with respect to HCV and HBV.

Hepatitis surface antigen (HBSAg) has been shown to be formed and assembled into particles in S. cerevisiae (Valenzuela et al. (1982)), as well as in, for example, mammalian cells (Valenzuela, P., et al. (1984)). formation of such particles has been shown to enhance the immunogenicity of the monomer subunit. The constructs may also include the immunodominant epitope of HBSAg, comprising the 55 amino acids of the presurface (pre-S) region. 30 Neurath et al. (1984). Constructs of the pre-S-HBSAg particle expressible in yeast are disclosed in EPO 174,444, published March 19, 1986; hybrids including heterologous viral sequences for yeast expression are disclosed in EPO 175,261, published March 26, 1966. Both applications are assigned to the herein assignee, and are

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incorporated herein by reference. These constructs may also be expressed in mammalian cells such as Chinese hamster ovary (CHO) cells using an SV40-dihydrofolate reductase vector (Michelle et al. (1984)).

In addition, portions of the particle-forming protein coding sequence may be replaced with codons encoding an HCV epitope. In this replacement, regions which are not required to mediate the aggregation of the units to form immunogenic particles in yeast or mammals can be deleted, thus eliminating additional HBV antigenic sites from competition with the HCV epitope.

II.E. Preparation of Vaccines

Vaccines may be prepared from one or more im-15 munogenic polypeptides derived from HCV cDNA as well as from the cDNA sequences in the Figs. 1-32, or from the HCV genome to which they correspond. The observed homology between HCV and Flaviviruses provides information concerning the polypeptides which are likely to be most 20 effective as vaccines, as well as the regions of the genome in which they are encoded. The general structure of the Flavivirus genome is discussed in Rice et al (1986). The flavivirus genomic RNA is believed to be the only virus-specific mRNA species, and it is translated 25 into the three viral structural proteins, i.e., C, M, and E, as well as two large nonstructural proteins, NV4 and NV5, and a complex set of smaller nonstructural proteins. It is known that major neutralizing epitopes for Flaviviruses reside in the E (envelope) protein (Roehrig 30 (1986)). The corresponding HCV E gene and polypeptide encoding region can be predicted, based upon the homology to Flaviviruses. Thus, vaccines may be comprised of recombinant polypeptides containing epitopes of HCV E. These polypeptides may be expressed in bacteria, yeast, or 35 mammalian cells, or alternatively may be isolated from

viral preparations. It is also anticipated that the other structural proteins may also contain epitopes which give rise to protective anti-HCV antibodies. Thus, polypeptides containing the epitopes of E, C, and M may also be used, whether singly or in combination, in HCV vaccines.

In addition to the above, it has been shown that immunization with NS1 (nonstructural protein 1), results in protection against yellow fever (Schlesinger et al (1986)). This is true even though the immunization does not give rise to neutralizing antibodies. Thus, particularly since this protein appears to be highly conserved among Flaviviruses, it is likely that HCV NS1 will also be protective against HCV infection. Moreover, it also shows that nonstructural proteins may provide protection against viral pathogenicity, even if they do not cause the production of neutralizing antibodies.

In view of the above, multivalent vaccines
against HCV may be comprised of one or more structural
proteins, and/or one or more nonstructural proteins.
These vaccines may be comprised of, for example,
recombinant HCV polypeptides and/or polypeptides isolated
from the virions. In addition, it may be possible to use
inactivated HCV in vaccines; inactivation may be by the
preparation of viral lysates, or by other means known in
the art to cause inactivation of Flaviviruses, for
example, treatment with organic solvents or detergents, or
treatment with formalin. Moreover, vaccines may also be
prepared from attenuated HCV strains. The preparation of
attenuated HCV strains is described infra.

It is known that some of the proteins in Flaviviruses contain highly conserved regions, thus, some immunological cross-reactivity is expected between HCV and other Flaviviruses. It is possible that shared epitopes between the Flaviviruses and HCV will give rise to

protective antibodies against one or more of the disorders caused by these pathogenic agents. Thus, it may be possible to design multipurpose vaccines based upon this knowledge.

The preparation of vaccines which contain an 5 immunogenic polypeptide(s) as active ingredients, is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or 10 suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active 15 ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering 20 agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine 25 (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-LalanyI-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-snglycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid 30 A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an HCV antigenic sequence resulting from administration of this polypeptide

in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutane-5 ously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or 10 triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, 15 magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or 25 phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

II.F. Dosage and Administration of Vaccines

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount 35

as will be prophylactically and/or therapeutically effec-The quantity to be administered, which is generally in the range of 5 micrograms to 250 micrograms of antigen per dose, depends on the subject to be treated, capacity 5 of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to 15 maintain and or reenforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment 20 of the practitioner.

In addition, the vaccine containing the immunogenic HCV antigen(s) may be administered in conjunction with other immunoregulatory agents, for example, immune globulins.

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The immunogenic polypeptides prepared as described above are used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are 30 desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an immunogenic polypeptide bearing an HCV epitope(s). Serum from the immunized

II.G. Preparation of Antibodies Against HCV Epitopes

procedures. If serum containing polyclonal antibodies to 35 an HCV epitope contains antibodies to other antigens, the

animal is collected and treated according to known

polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987).

Alternatively, polyclonal antibodies may be isolated from a mammal which has been previously infected with HCV. An example of a method for purifying antibodies to HCV epitopes from serum from an infected individual, based upon affinity chromatography and utilizing a fusion 10 polypeptide of SOD and a polypeptide encoded within cDNA clone 5-1-1, is presented in Section V.E.

Monoclonal antibodies directed against HCV epitopes can also be readily produced by one skilled in The general methodology for making monoclonal 15 antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. 20 Schreier et al. (1980); Hammerling et al. (1981); Kennett et al. (1980); see also, U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against HCV epitopes can be screened for various 25 properties; i.e., for isotype, epitope affinity, etc.

Antibodies, both monoclonal and polyclonal, which are directed against HCV epitopes are particularly useful in diagnosis, and those which are neutralizing are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies.

Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired.

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See, for example, Nisonoff, A., et al. (1981) and Dreesman et al. (1985).

Techniques for raising anti-idiotype antibodies are known in the art. See, for example, Grzych (1985),

MacNamara et al. (1984), and Uytdehaag et al. (1985).

These anti-idiotype antibodies may also be useful for treatment of NANBH, as well as for an elucidation of the immunogenic regions of HCV antigens.

II.H. Diagnostic Oligonucleotide Probes and Kits

Using the disclosed portions of the isolated HCV cDNAs as a basis, including those in Figs. 1-32, oligomers of approximately 8 nucleotides or more can be prepared, either by excision or synthetically, which hybridize with the HCV genome and are useful in identification of the viral agent(s), further characterization of the viral genome(s), as well as in detection of the virus(es) in diseased individuals. The probes for HCV polynucleotides (natural or derived) are a length which allows the detection of unique viral sequences by hybridization. While 6-8 nucleotides may be a workable length, sequences of 10-12 nucleotides are preferred, and about 20 nucleotides appears optimal. Preferably, these sequences will derive from regions which lack heterogeneity. probes can be prepared using routine methods, including automated oligonucleotide synthetic methods. Among useful probes, for example, are the clone 5-1-1 and the additional clones disclosed herein, as well as the various oligomers useful in probing cDNA libraries, set forth below. A complement to any unique portion of the HCV genome will be satisfactory. For use as probes, complete complementarity is desirable, though it may be unnecessary as the length of the fragment is increased.

For use of such probes as diagnostics, the biological sample to be analyzed, such as blood or serum, is

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treated, if desired, to extract the nucleic acids contained therein. The resulting nucleic acid from the sample may be subjected to gel electrophoresis or other size separation techniques; alternatively, the nucleic acid sample may be dot blotted without size separation. The probes are then labeled. Suitable labels, and methods for labeling probes are known in the art, and include, for example, radioactive labels incorporated by nick translation or kinasing, biotin, fluorescent probes, and chemiluminescent probes. The nucleic acids extracted from the sample are then treated with the labeled probe under hybridization conditions of suitable stringencies.

The probes can be made completely complementary to the HCV genome. Therefore, usually high stringency conditions are desirable in order to prevent false positives. However, conditions of high stringency should only be used if the probes are complementary to regions of the viral genome which lack heterogeneity. The stringency of hybridization is determined by a number of factors during hybridization and during the washing procedure, including temperature, ionic strength, length of time, and concentration of formamide. These factors are outlined in, for example, Maniatis, T. (1982).

Generally, it is expected that the HCV genome sequences will be present in serum of infected individuals at relatively low levels, i.e., at approximately 10^2-10^3 sequences per ml. This level may require that amplification techniques be used in hybridization assays. Such techniques are known in the art. For example, the Enzo Biochemical Corporation "Bio-Bridge" system uses terminal deoxynucleotide transferase to add unmodified 3'-poly-dTtails to a DNA probe. The poly dT-tailed probe is hybridized to the target nucleotide sequence, and then to a biotin-modified poly-A. PCT application 84/03520 and EPA124221 describe a DNA hybridization assay in which: (1)

analyte is annealed to a single-stranded DNA probe that is complementary to an enzyme-labeled oligonucleotide; and (2) the resulting tailed duplex is hybridized to an enzyme-labeled oligonucleotide. EPA 204510 describes a 5 DNA hybridization assay in which analyte DNA is contacted with a probe that has a tail, such as a poly-dT tail, an amplifier strand that has a sequence that hybridizes to the tail of the probe, such as a poly-A sequence, and which is capable of binding a plurality of labeled 10 strands. A particularly desirable technique may first involve amplification of the target HCV sequences in sera approximately 10,000 fold, i.e., to approximately 10° sequences/ml. This may be accomplished, for example, by the technique of Saiki et al. (1986). The amplified 15 sequence(s) may then be detected using a hybridization assay which is described in co-pending U.S. Application, Attorney Docket No. 2300-0171, which was filed 15 October 1987, is assigned to the herein assignee, and is hereby incorporated herein by reference. This hybridization as-20 say, which should detect sequences at the level of 10 ml utilizes nucleic acid multimers which bind to singlestranded analyte nucleic acid, and which also bind to a multiplicity of single-stranded labeled oligonucleotides. A suitable solution phase sandwich assay which may be used 25 with labeled polynucleotide probes, and the methods for the preparation of probes is described in EPO 225,807, published June 16, 1987, which is assigned to the herein assignee, and which is hereby incorporated herein by reference.

The probes can be packaged into diagnostic kits.

Diagnostic kits include the probe DNA, which may be labeled; alternatively, the probe DNA may be unlabeled and the ingredients for labeling may be included in the kit.

The kit may also contain other suitably packaged reagents and materials needed for the particular hybridization

protocol, for example, standards, as well as instructions for conducting the test.

II.I. Immunoassay and Diagnostic Kits

Both the polypeptides which react immuno-5 logically with serum containing HCV antibodies, for example, those derived from or encoded within the clones described in Section IV.A., and composites thereof, (see section IV.A.) and the antibodies raised against the HCV 10 specific epitopes in these polypeptides, see for example Section IV.E, are useful in immunoassays to detect presence of HCV antibodies, or the presence of the virus and/or viral antigens, in biological samples, including for example, blood or serum samples. Design of the 15 immunoassays is subject to a great deal of variation, and a variety of these are known in the art. For example, the immunoassay may utilize one viral antigen, for example, a polypeptide derived from any of the clones containing HCV cDNA described in Section IV.A., or from the composite 20 cDNAs derived from the cDNAs in these clones, or from the HCV genome from which the cDNA in these clones is derived; alternatively, the immunoassay may use a combination of viral antigens derived from these sources. for example, a monoclonal antibody directed towards a 25 viral epitope(s), a combination of monoclonal antibodies directed towards one viral antigen, monoclonal antibodies directed towards different viral antigens, polyclonal antibodies directed towards the same viral antigen, or polyclonal antibodies directed towards different viral antigens. Protocols may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, fluorescent, chemiluminescent, radioactive,

or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

The Flavivirus model for HCV allows predictions regarding the likely location of diagnostic epitopes for the virion structural proteins. The C, pre-M, M, and E domains are all likely to contain epitopes of significant potential for detecting viral antigens, and particularly for diagnosis. Similarly, domains of the nonstructural proteins are expected to contain important diagnostic epitopes (e.g., NS5 encoding a putative polymerase; and NS1 encoding a putative complement-binding antigen). Recombinant polypeptides, or viral polypeptides, which include epitopes from these specific domains may be useful for the detection of viral antibodies in infections blood donors and infected patients.

In addition, antibodies directed against the E and/or M proteins can be used in immunoassays for the detection of viral antigens in patients with HCV caused NANBH, and in infectious blood donors. Moreover, these antibodies will be extremely useful in detecting acutephase donors and patients.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by
packaging the appropriate materials, including the
polypeptides of the invention containing HCV epitopes or
antibodies directed against HCV epitopes in suitable
containers, along with the remaining reagents and materials required for the conduct of the assay, as well as a
suitable set of assay instructions.

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II.J. Further Characterization of the HCV Genome,
Virions, and Viral Antigens Using Probes Derived From cDNA
to the Viral Genome

The HCV cDNA sequence information in the clones

described in Section IV.A., as shown in Figs. 1-32,
inclusive, may be used to gain further information on the
sequence of the HCV genome, and for identification and
isolation of the HCV agent, and thus will aid in its
characterization including the nature of the genome, the

structure of the viral particle, and the nature of the
antigens of which it is composed. This information, in
turn, can lead to additional polynucleotide probes,
polypeptides derived from the HCV genome, and antibodies
directed against HCV epitopes which would be useful for
the diagnosis and/or treatment of HCV caused NANBH.

The cDNA sequence information in the abovementioned clones is useful for the design of probes for the isolation of additional cDNA sequences which are derived from as yet undefined regions of the HCV genome(s) 20 from which the cDNAs in clones described in Section IV.A. are derived. For example, labeled probes containing a sequence of approximately 8 or more nucleotides, and preferably 20 or more nucleotides, which are derived from regions close to the 5'-termini or 3'-termini of the fam-25 ily of HCV cDNA sequences shown in Figs. 1, 3, 6, 9, 14 and 32 may be used to isolate overlapping cDNA sequences from HCV cDNA libraries. These sequences which overlap the cDNAs in the above-mentioned clones, but which also contain sequences derived from regions of the genome from 30 which the cDNA in the above mentioned clones are not derived, may then be used to synthesize probes for identification of other overlapping fragments which do not necessarily overlap the cDNAs in the clones described in Section IV.A. Unless the HCV genome is segmented and the 35 segments lack common sequences, it is possible to sequence

the entire viral genome(s) utilizing the technique of isolation of overlapping cDNAs derived from the viral genome(s). Although it is unlikely, if the genome is a segmented genome which lacks common sequences, the 5 sequence of the genome can be determined by serologically screening lambda-gtl1 HCV cDNA libraries, as used to isolate clone 5-1-1, sequencing cDNA isolates, and using the isolated cDNAs to isolate overlapping fragments, using the technique described for the isolation and sequencing 10 of the clones described in Section IV.A. Alternatively, characterization of the genomic segments could be from the viral genome(s) isolated from purified HCV particles. Methods for purifying HCV particles and for detecting them during the purification procedure are described herein, 15 infra. Procedures for isolating polynucleotide genomes from viral particles are known in the art, and one procedure which may be used is shown in Example IV.A.1. The isolated genomic segments could then be cloned and sequenced. Thus, with the information provided herein, it 20 is possible to clone and sequence the HCV genome(s) irrespective of their nature.

Methods for constructing cDNA libraries are known in the art, and are discussed supra and infra; a method for the construction of HCV cDNA libraries in

25 lambda-gtll is discussed infra in Section IV.A. However, cDNA libraries which are useful for screening with nucleic acid probes may also be constructed in other vectors known in the art, for example, lambda-gtl0 (Huynh et al. (1985)). The HCV derived cDNA detected by the probes derived from the cDNAs in Figs. 1-32, and from the probes synthesized from polynucleotides derived from these cDNAs, may be isolated from the clone by digestion of the isolated polynucleotide with the appropriate restriction enzyme(s), and sequenced. See, for example, Section

35 IV.A.3. and IV.A.4. for the techniques used for the

isolation and sequencing of HCV cDNA which overlaps HCV cDNA in clone 5-1-1, Sections IV.A.5-IV.A.7 for the isolation and sequencing of HCV cDNA which overlaps that in clone 81, and Section IV.A.8 and IV.A.9 for the isolation and sequencing of a clone which overlaps another clone (clone 36), which overlaps clone 81.

The sequence information derived from these overlapping HCV cDNAs is useful for determining areas of homology and heterogeneity within the viral genome(s), 10 which could indicate the presence of different strains of the genome, and/or of populations of defective particles. It is also useful for the design of hybridization probes to detect HCV or HCV antigens or HCV nucleic acids in biological samples, and during the isolation of HCV 15 (discussed infra), utilizing the techniques described in Section II.G. Moreover, the overlapping cDNAs may be used to create expression vectors for polypeptides derived from the HCV genome(s) which also encode the polypeptides encoded in clones 5-1-1, 36, 81, 91, and 1-2, and in the 20 other clones described in Section IV.A. The techniques for the creation of these polypeptides containing HCV epitopes, and for antibodies directed against HCV epitopes contained within them, as well as their uses, are analogous to those described for polypeptides derived from 25 NANBV cDNA sequences contained within clones 5-1-1, 32, 35, 36, 1-2, 81, and 91, discussed supra and infra. Encoded within the family of cDNA sequences

contained within the family of CDNA sequences contained within clones 5-1-1, 32, 35, 36, 81, 91, 1-2, and the other clones described in Section IV.A. are antigen(s) containing epitopes which appear to be unique to HCV; i.e., antibodies directed against these antigens are absent from individuals infected with HAV or HBV, and from individuals not infected with HCV (see the serological data presented in Section IV.B.). Moreover, a comparison of the sequence information of these cDNAs with

the sequences of HAV, HBV, HDV, and with the genomic sequences in Genebank indicates that minimal homology exists between these cDNAs and the polynucleotide sequences of those sources. Thus, antibodies directed against the antigens encoded within the cDNAs of these clones may be used to identify BB-NANBV particles isolated from infected individuals. In addition, they are also useful for the isolation of NANBH agent(s).

HCV particles may be isolated from the sera from 10 BB-NANBV infected individuals or from cell cultures by any of the methods known in the art, including for example, techniques based on size discrimination such as sedimentation or exclusion methods, or techniques based on density such as ultracentrifugation in density gradients, or 15 precipitation with agents such as polyethylene glycol, or chromatography on a variety of materials such as anionic or cationic exchange materials, and materials which bind due to hydrophobicity, as well as affinity columns. ing the isolation procedure the presence of HCV may be 20 detected by hybridization analysis of the extracted genome, using probes derived from the HCV cDNAs described supra, or by immunoassay (see Section II.I.) utilizing as probes antibodies directed against HCV antigens encoded within the family of cDNA sequences shown in Figs. 1-32, 25 and also directed against HCV antigens encoded within the overlapping HCV cDNA sequences discussed supra. The antibodies may be monoclonal, or polyclonal, and it may be desirable to purify the antibodies before their use in the immunoassay. A purification procedure for polyclonal 30 antibodies directed against antigen(s) encoded within clone 5-1-1 is described in Section IV.E; analogous purification procedures may be utilized for antibodies directed against other HCV antigens.

Antibodies directed against HCV antigens encoded 35 within the family of cDNAs shown in Figs. 1-32, as well as

those encoded within overlapping HCV cDNAs, which are affixed to solid supports are useful for the isolation of HCV by immunoaffinity chromatography. Techniques for immunoaffinity chromatography are known in the art, 5 including techniques for affixing antibodies to solid supports so that they retain their immunoselective activity; the techniques may be those in which the antibodies are adsorbed to the support (see, for example, Kurstak in ENZYME IMMUNODIAGNOSIS, page 31-37), as well as those in 10 which the antibodies are covalently linked to the support. Generally, the techniques are similar to those used for covalent linking of antigens to a solid support, which are generally described in Section II.C.; however, spacer groups may be included in the bifunctional coupling agents so that the antigen binding site of the antibody remains accessible.

During the purification procedure the presence of HCV may be detected and/or verified by nucleic acid hybridization, utilizing as probes polynucleotides derived 20 from the family of HCV cDNA sequences shown in Figs. 1-32, as well as from overlapping HCV cDNA sequences, described supra. In this case, the fractions are treated under conditions which would cause the disruption of viral particles, for example, with detergents in the presence of 25 chelating agents, and the presence of viral nucleic acid determined by hybridization techniques described in Section II.H. .Further confirmation that the isolated particles are the agents which induce HCV may be obtained by infecting chimpanzees with the isolated virus 30 particles, followed by a determination of whether the symptoms of NANBH result from the infection.

Viral particles from the purified preparations may then be further characterized. The genomic nucleic acid has been purified. Based upon its sensitivity to` RNase, and not DNase I, it appears that the virus is

composed of an RNA genome. See Example IV.C.2., infra. The strandedness and circularity or non-circularity can determined by techniques known in the art, including, for example, its visualization by electron microscopy, its 5 migration in density gradients, and its sedimentation characteristics. Based upon the hybridization of the captured HCV genome to the negative strands of HCV cDNAs, it appears that HCV may be comprised of a positive stranded RNA genome (see Section IV.H.1). Techniques such 10 as these are described in, for example, METHODS IN ENZYMOLOGY. In addition, the purified nucleic acid can be cloned and sequenced by known techniques, including reverse transcription since the genomic material is RNA. See, for example, Maniatis (1982), and Glover (1985). 15 Utilizing the nucleic acid derived from the viral particles, it is possible to sequence the entire genome, whether or not it is segmented.

Examination of the homology of the polypeptide encoded within the continuous ORF of combined clones 14i 20 through 39c (see Fig. 26), shows that the HCV polypeptide contains regions of homology with the corresponding proteins in conserved regions of flaviviruses. An example of this is described in Section IV.H.3. This finding has many important ramifications. First, this evidence, in 25 conjunction with the results which show that HCV contains a positive-stranded genome, the size of which is approximately 10,000 nucleotides, is consistent with the suggestion that HCV is a flavivirus, or flavi-like virus. Generally, flavivirus virions and their genomes have a 30 relatively consistent structure and organization, which See Rice et al. (1986), and Brinton, M.A. are known. (1988). Thus, the structural genes encoding the polypeptides C, pre-M/M, and E may be located in the 5 %terminus of the genome upstream of clone 14i. Moreover, 35 using the comparison with other flaviviruses, predictions

as to the precise location of the sequences encoding these proteins can be made.

Isolation of the sequences upstream of those in clone 14i may be accomplished in a number of ways which, 5 given the information herein, would be obvious to one of skill in the art. For example, the genome "walking" technique, may be used to isolate other sequences which are 5' to those in clone 14i, but which overlap that clone; this in turn leads to the isolation of additional sequences. This technique has been amply demonstrated infra, in Section IV.A.. For example, also, it is known that the flaviviruses have conserved epitopes and regions of conserved nucleic acid sequences. Polynucleotides containing the conserved sequences may be used as probes 15 which bind the HCV genome, thus allowing its isolation. In addition, these conserved sequences, in conjunction with those derived from the HCV cDNAs shown in Fig. 22, may be used to design primers for use in systems which amplify the genome sequences upstream of those in clone 14i, using polymerase chain reaction technology. An example of this is described infra.

The structure of the HCV may also be determined and its components isolated. The morphology and size may be determined by, for example, electron microscopy. 25 identification and localization of specific viral polypeptide antigens such as coat or envelope antigens, or internal antigens, such as nucleic acid binding proteins, core antigens, and polynucleotide polymerase(s) may also be determined by, for example, determining whether the 30 antigens are present as major or minor viral components, as well as by utilizing antibodies directed against the specific antigens encoded within isolated cDNAs as probes. This information is useful in the design of vaccines; for example, it may be preferable to include an exterior antigen in a vaccine preparation. Multivalent vaccines

may be comprised of, for example, a polypeptide derived from the genome encoding a structural protein, for example, E, as well as a polypeptide from another portion of the genome, for example, a nonstructural or structural polypeptide.

II.K. <u>Cell Culture Systems and Animal Model Systems for</u> HCV Replication

The suggestion that HCV is a flavivirus or 10 flavi-like virus also provides information on methods for growing HCV. The term "flavi-like" means that the virus shows a significant amount of homology to the known conserved regions of flaviviruses and that the majority of the genome is a single ORF. Methods for culturing 15 flaviviruses are known to those of skill in the art (See, for example, the reviews by Brinton (1986) and Stollar, V. (1980)). Generally, suitable cells or cell lines for culturing HCV may include those known to support Flavivirus replication, for example, the following: monkey 20 kidney cell lines (e.g. MK2, VERO); porcine kidney cell lines (e.g. PS); baby hamster kidney cell lines (e.g. BHK); murine macrophage cell lines (e.g., P388D1, MK1, Mm1); human macrophage cell lines (e.g., U-937); human peripheral blood leukocytes; human adherent monocytes; 25 hepatocytes or hepatocyte cell lines (e.g., HUH7 , HEPG2); embryos or embryonic cells (e.g., chick embryo fibroblasts); or cell lines derived from invertebrates, preferably from insects (e.g. drosophila cell lines), or more preferably from arthropods, for example, mosquito 30 cell lines (e.g., A. Albopictus, Aedes aegypti, Cutex tritaeniorhynchus) or tick cell lines (e.g. RML-14 Dermacentor parumapertus).

It is possible that primary hepatocytes can be cultured, and then infected with HCV; or alternatively, the hepatocyte cultures could be derived from the livers

of infected individuals (e.g., humans or chimpanzees). The latter case is an example of a cell which is infected in vivo being passaged in vitro. In addition, various immortalization methods can be used to obtain cell-lines 5 derived from hepatocyte cultures. For example, primary liver cultures (before and after enrichment of the hepatocyte population) may be fused to a variety of cells to maintain stability. For example, also, cultures may be infected with transforming viruses, or transfected with 10 transforming genes in order to create permanent or semipermanent cell lines. In addition, for example, cells in liver cultures may be fused to established cell lines (e.g., HepG2). Methods for cell fusion are known in the art, and include, for example, the use of fusion agents 15 such as polyethylene glycol, Sendai Virus, and Epstein-Barr virus.

As discussed above, HCV is a Flavivirus or Flavi-like virus. Therefore, it is probable that HCV infection of cell lines may be accomplished by techniques 20 known in the art for infecting cells with Flaviviruses. These include, for example, incubating the cells with viral preparations under conditions which allow viral entry into the cell. In addition, it may be possible to obtain viral production by transfecting the cells with 25 isolated viral polynucleotides. It is known that Togavirus and Flavivirus RNAs are infectious in a variety of vertebrate cell lines (Pfefferkorn and Shapiro (1974)), and in a mosquito cell line (Peleg (1969)). Methods for transfecting tissue culture cells with RNA 30 duplexes, positive stranded RNAs, and DNAs (including cDNAs) are known in the art, and include, for example, techniques which use electroporation, and precipitation with DEAE-Dextran or calcium phosphate. An abundant source of HCV RNA can be obtained by performing in vitro 35 transcription of an HCV cDNA corresponding to the complete genome. Transfection with this material, or with cloned HCV cDNA should result in viral replication and the $\underline{\text{in}}$ $\underline{\text{vitro}}$ propagation of the virus.

In addition to cultured cells, animal model

5 systems may be used for viral replication; animal systems in which flaviviruses are known to those of skill in the art (See, for example, the review by Monath (1986)).

Thus, HCV replication may occur not only in chimpanzees, but also in, for example, marmosets and suckling mice.

10

II.L. Screening for Anti-Viral Agents for HCV

The availability of cell culture and animal model systems for HCV also makes possible screening for anti-viral agents which inhibit HCV replication, and particularly for those agents which preferentially allow cell growth and multiplication while inhibiting viral replication. These screening methods are known by those of skill in the art. Generally, the anti-viral agents are tested at a variety of concentrations, for their effect on preventing viral replication in cell culture systems which support viral replication, and then for an inhibition of infectivity or of viral pathogenicity (and a low level of toxicity) in an animal model system.

The methods and compositions provided herein for detecting HCV antigens and HCV polynucleotides are useful for screening of anti-viral agents in that they provide an alternative, and perhaps more sensitive means, for detecting the agent's effect on viral replication than the cell plaque assay or ID₅₀ assay. For example, the HCV-polynucleotide probes described herein may be used to quantitate the amount of viral nucleic acid produced in a cell culture. This could be accomplished, for example, by hybridization or competition hybridization of the infected cell nucleic acids with a labeled HCV-polynucleotide probe. For example, also, anti-HCV antibodies may be used

to identify and quantitate HCV antigen(s) in the cell culture utilizing the immunoassays described herein. In addition, since it may be desirable to quantitate HCV antigens in the infected cell culture by a competition assay, the polypeptides encoded within the HCV cDNAs described herein are useful in these competition assays. Generally, a recombinant HCV polypeptide derived from the HCV cDNA would be labeled, and the inhibition of binding of this labeled polypeptide to an HCV polypeptide due to the antigen produced in the cell culture system would be monitored. Moreover, these techniques are particularly useful in cases where the HCV may be able to replicate in a cell line without causing cell death.

15 II.M. Preparation of Attenuated Strains of HCV

In addition to the above, utilizing the tissue culture systems and/or animal model systems, it may be possible to isolate attenuated strains of HCV. strains would be suitable for vaccines, or for the isola-20 tion of viral antigens. Attenuated strains are isolatable after multiple passages in cell culture and/or an animal model. Detection of an attenuated strain in an infected cell or individual is achievable by techniques known in the art, and could include, for example, the use of 25 antibodies to one or more epitopes encoded in HCV as a probe or the use of a polynucleotide containing an HCV sequence of at least about 8 nucleotides as a probe. Alternatively, or in addition, an attenuated strain may be constructed utilizing the genomic information of HCV 30 provided herein, and utilizing recombinant techniques. Generally, one would attempt to delete a region of the genome encoding, for example, a polypeptide related to pathogenicity, but which allows viral replication. addition, the genome construction would allow the

35 expression of an epitope which gives rise to neutralizing

antibodies for HCV. The altered genome could then be utilized to transform cells which allow HCV replication, and the cells grown under conditions to allow viral replication. Attenuated HCV strains are useful not only for vaccine purposes, but also as sources for the commercial production of viral antigens, since the processing of these viruses would require less stringent protection measures for the employees involved in viral production and/or the production of viral products.

10

III. General Methods

The general techniques used in extracting the genome from a virus, preparing and probing a cDNA library, sequencing clones, constructing expression vectors, transforming cells, performing immunological assays such as radioimmunoassays and ELISA assays, for growing cells in culture, and the like are known in the art and laboratory manuals are available describing these techniques. However, as a general guide, the following sets forth some sources currently available for such procedures, and for materials useful in carrying them out.

III.A. Hosts and Expression Control Sequences

Both prokaryotic and eukaryotic host cells may

25 be used for expression of desired coding sequences when
appropriate control sequences which are compatible with
the designated host are used. Among prokaryotic hosts, E.
coli is most frequently used. Expression control
sequences for prokaryotes include promoters, optionally

30 containing operator portions, and ribosome binding sites.
Transfer vectors compatible with prokaryotic hosts are
commonly derived from, for example, pBR322, a plasmid
containing operons conferring ampicillin and tetracycline
resistance, and the various pUC vectors, which also
35 contain sequences conferring antibiotic resistance mark-

ers. These markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include the Beta-lactamase (penicillinase) and lactose promoter systems (Chang et al. (1977)), the tryptophan (trp) promoter system (Goeddel et al. (1980)) and the lambda-derived P_L promoter and N gene ribosome binding site (Shimatake et al. (1981)) and the hybrid tac promoter (De Boer et al. (1983)) derived from sequences of the trp and lac UV5 promoters. The foregoing systems are particularly compatible with E. coli; if desired, other prokaryotic hosts such as strains of Bacillus or Pseudomonas may be used, with corresponding control sequences.

Eukaryotic hosts include yeast and mammalian 15 cells in culture systems. Saccharomyces cerevisiae and Saccharomyces carlsbergensis are the most commonly used yeast hosts, and are convenient fungal hosts. Yeast compatible vectors carry markers which permit selection of successful transformants by conferring prototrophy to 20 auxotrophic mutants or resistance to heavy metals on wildtype strains. Yeast compatible vectors may employ the 2 micron origin of replication (Broach et al. (1983)), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences which will result in in-25 corporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors are known in the art and include promoters for the synthesis of glycolytic enzymes (Hess et al. (1968); Holland et al. (1978)), including the promoter for 3 phosphoglycerate 30 kinase (Hitzeman (1980)). Terminators may also be included, such as those derived from the enolase gene (Holland (1981)). Particularly useful control systems are those which comprise the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from

GAPDH, and if secretion is desired, leader sequence from yeast alpha factor. In addition, the transcriptional regulatory region and the transcriptional initiation region which are operably linked may be such that they are not naturally associated in the wild-type organism. These systems are described in detail in EPO 120,551, published October 3, 1984; EPO 116,201, published August 22, 1984; and EPO 164,556, published December 18, 1985, all of which are assigned to the herein assignee, and are hereby incorporated herein by reference.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including HeLa cells, Chinese hamster 15 ovary (CHO) cells, baby hamster kidney (BHK) cells, and a number of other cell lines. Suitable promoters for mammalian cells are also known in the art and include viral promoters such as that from Simian Virus 40 (SV40) (Fiers (1978)), Rous sarcoma virus (RSV), adenovirus (ADV), and 20 bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences 25 are known in the art. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which insure integration of the appropriate sequences encoding NANBV epitopes into the host genome.

30 III.B. Transformations

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Transformation may be by any known method for introducing polynucleotides into a host cell, including, for example packaging the polynucleotide in a virus and transducing a host cell with the virus, and by direct uptake of the polynucleotide. The transformation

procedure used depends upon the host to be transformed:
For example, transformation of the E. coli host cells with lambda-gtll containing BB-NANBV sequences is discussed in the Example section, infra. Bacterial transformation by direct uptake generally employs treatment with calcium or rubidium chloride (Cohen (1972); Maniatis (1982)). Yeast transformation by direct uptake may be carried out using the method of Hinnen et al. (1978). Mammalian transformations by direct uptake may be conducted using the calcium phosphate precipitation method of Graham and Van der Eb (1978), or the various known modifications thereof.

III.C. <u>Vector Construction</u>

Vector construction employs techniques which are known in the art. Site-specific DNA cleavage is performed by treating with suitable restriction enzymes under conditions which generally are specified by the manufacturer of these commercially available enzymes. In general, about 1 microgram of plasmid or DNA sequence is cleaved by 1 unit of enzyme in about 20 microliters buffer solution by incubation of 1-2 hr at 37° C. After incubation with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved fragments may be separated using polyacrylamide or agarose gel electrophoresis techniques, according to the general procedures found in Methods in Enzymology (1980) 65:499-560.

Sticky ended cleavage fragments may be blunt ended using <u>E. coli</u> DNA polymerase I (Klenow) in the pressure of the appropriate deoxynucleotide triphosphates (dNTPs) present in the mixture. Treatment with S1 nuclease may also be used, resulting in the hydrolysis of any single stranded DNA portions.

Ligations are carried out using standard buffer and temperature conditions using T4 DNA ligase and ATP;

sticky end ligations require less ATP and less ligase than blunt end ligations. When vector fragments are used-as part of a ligation mixture, the vector fragment is often treated with bacterial alkaline phosphatase (BAP) or calf 5 intestinal alkaline phosphatase to remove the 5'-phosphate and thus prevent religation of the vector; alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent ligation.

Ligation mixtures are transformed into suitable 10 cloning hosts, such as E. coli, and successful transformants selected by, for example, antibiotic resistance, and screened for the correct construction.

III.D. Construction of Desired DNA Sequences

Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer as described by Warner (1984). If desired the synthetic strands may be labeled with ³²P by treatment with polynucleotide kinase in the presence of ³²P-ATP, using standard conditions for 20 the reaction.

DNA sequences, including those isolated from cDNA libraries, may be modified by known techniques, including, for example site directed mutagenesis, as described by Zoller (1982). Briefly, the DNA to be 25 modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own 30 sequence. The resulting double stranded DNA is transformed into a phage supporting host bacterium. Cultures of the transformed bacteria, which contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically, 50% of the new 35 plaques contain phage having the mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to labeled synthetic probe at temperatures and conditions which permit hybridization with the correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization are recovered and cloned.

III.E. Hybridization with Probe

DNA libraries may be probed using the procedure 10 of Grunstein and Hogness (1975). Briefly, in this procedure, the DNA to be probed is immobilized on nitrocellulose filters, denatured, and prehybridized with a buffer containing 0-50% formamide, 0.75 M NaCl, 75 mM Na citrate, 0.02% (wt/v) each of bovine serum albumin, poly-15 vinyl pyrollidone, and Ficoll, 50 mM Na Phosphate (pH 6.5), 0.1% SDS, and 100 micrograms/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps 20 depends on the stringency required. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides such as those 25 derived from cDNA or genomic sequences generally employ higher temperatures, e.g., about $40-42^{\circ}\text{C}$, and a high percentage, e.g., 50%, formamide. Following prehybridization, 5'-32P-labeled oligonucleotide probe is added to the buffer, and the filters are incubated in this 30 mixture under hybridization conditions. After washing, the treated filters are subjected to autoradiography to show the location of the hybridized probe; DNA in corresponding locations on the original agar plates is used as the source of the desired DNA.

III.F. Verification of Construction and Sequencing

For routine vector constructions, ligation mixtures are transformed into <u>E</u>. <u>coli</u> strain HB101 or other suitable host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants are then prepared according to the method of Clewell et al. (1969), usually following chloramphenicol amplification (Clewell (1972)). The DNA is isolated and analyzed, usually by restriction enzyme analysis and/or sequencing. Sequencing may be by the dideoxy method of Sanger et al. (1977) as further described by Messing et al. (1981), or by the method of Maxam et al. (1980). Problems with band compression, which are sometimes observed in GC rich regions, were overcome by use of T-deazoguanosine according to Barr et al. (1986).

III.G. Enzyme Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) 20 can be used to measure either antigen or antibody concentrations. This method depends upon conjugation of an enzyme to either an antigen or an antibody, and uses the bound enzyme activity as a quantitative label. measure antibody, the known antigen is fixed to a solid 25 phase (e.g., a microplate or plastic cup), incubated with test serum dilutions, washed, incubated with antiimmunoglobulin labeled with an enzyme, and washed again. Enzymes suitable for labeling are known in the art, and include, for example, horseradish peroxidase. Enzyme 30 activity bound to the solid phase is measured by adding the specific substrate, and determining product formation or substrate utilization colorimetrically. The enzyme activity bound is a direct function of the amount of antibody bound.

To measure antigen, a known specific antibody is fixed to the solid phase, the test material containing antigen is added, after an incubation the solid phase is washed, and a second enzyme-labeled antibody is added.

5 After washing, substrate is added, and enzyme activity is estimated colorimetrically, and related to antigen concentration.

IV. Examples

Described below are examples of the present 10 invention which are provided only for illustrative purposes, and not to limit the scope of the present inven-In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art. The procedures 15 set forth, for example, in Sections IV.A. may, if desired, be repeated but need not be, as techniques are available for construction of the desired nucleotide sequences based on the information provided by the invention. Expression is exemplified in E. coli; however, other systems are available as set forth more fully in Section III.A. ditional epitopes derived from the genomic structure may also be produced, and used to generate antibodies as set forth below.

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IV.A. Preparation, Isolation and Sequencing of HCV cDNA

IV.A.1. Preparation of HCV cDNA

The source of NANB agent was a plasma pool

derived from a chimpanzee with chronic NANBH. The
chimpanzee had been experimentally infected with blood
from another chimpanzee with chronic NANBH resulting from
infection with HCV in a contaminated batch of factor 8
concentrate derived from pooled human sera. The

chimpanzee plasma pool was made by combining many

individual plasma samples containing high levels of alanine aminotransferase activity; this activity results from hepatic injury due to the HCV infection. Since 1 ml of a 10⁻⁶ dilution of this pooled serum given i.v. caused NANBH in another chimpanzee, its CID was at least 10⁶/ml, i.e., it had a high infectious virus titer.

A cDNA library from the high titer plasma pool was generated as follows. First, viral particles were isolated from the plasma; a 90 ml aliquot was diluted with 310 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 10 1mM EDTA, 100 mM NaCl. Debris was removed by centrifugation for 20 min at 15,000 x g at 20°C. Viral particles in the resulting supernatant were then pelleted by centrifugation in a Beckman SW28 rotor at 28,000 rpm for 5 15 hours at 20°C. To release the viral genome, the particles were disrupted by suspending the pellets in 15 ml solution containing 1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 10 mM Tris-HCl, pH 7.5, also containing 2 mg/ml proteinase k, followed by incubation at 45°C for 90 min. Nucleic acids 20 were isolated by adding 0.8 micrograms MS2 bacteriophage RNA as carrier, and extracting the mixture four times with a 1:1 mixture of phenol:chloroform (phenol saturated with 0.5M Tris-HCl, pH 7.5, 0.1% (v/v) beta-mercaptoethanol, 0.1% (w/v) hydroxyquinolone, followed by extraction two 25 times with chloroform. The aqueous phase was concentrated with 1-butanol prior to precipitation with 2.5 volumes absolute ethanol overnight at -20°C. Nucleic acid was recovered by centrifugation in a Beckman SW41 rotor at 40,000 rpm for 90 min at 40°C, and dissolved in water that 30 had been treated with 0.05% (v/v) diethylpyrocarbonate and autoclaved.

Nucleic acid obtained by the above procedure (<2 micrograms) was denatured with 17.5 mM CH₃HgOH; cDNA was synthesized using this denatured nucleic acid as template, and was cloned into the EcoRI site of phage lambda-gtl1

using methods described by Huynh (1985), except that random primers replaced oligo(dT) 12-18 during the synthesis of the first cDNA strand by reverse transcriptase (Taylor et al. (1976)). The resulting 5 double stranded cDNAs were fractionated according to size on a Sepharose CL-4B column; eluted material of approximate mean size 400, 300, 200, and 100 base-pairs were pooled into cDNA pools 1, 2, 3, and 4, respectively. The lambda-gtll cDNA library was generated from the cDNA in pool 3.

The lambda-gt11 cDNA library generated from pool 3 was screened for epitopes that could bind specifically with serum derived from a patient who had previously experienced NANBH. About 10⁶ phage were screened with patient sera using the methods of Huynh et al. (1985), except that bound human antibody was detected with sheep anti-human Ig antisera that had been radio-labeled with Five positive phages were identified and purified. The five positive phages were then tested for specificity of binding to sera from 8 different humans previously infected with the NANBH agent, using the same method. Four of the phage encoded a polypeptide that reacted immunologically with only one human serum, i.e., the one that was used for primary screening of the phage library. The fifth phage (5-1-1) encoded a polypeptide that reacted immunologically with 5 of 8 of the sera tested. Moreover, this polypeptide did not react immunologically with sera from 7 normal blood donors. Therefore, it appears that clone 5-1-1 encodes a polypeptide which is specifically 30 recognized immunologically by sera from NANB patients.

IV.A.2. Sequences of the HCV cDNA in Recombinant Phage 5-1-1, and of the Polypeptide Encoded Within the Sequence.

The cDNA in recombinant phage 5-1-1 was 35 sequenced by the method of Sanger et al. (1977). Essentially, the cDNA was excised with EcoRI, isolated by size fractionation using gel electrophoresis. The EcoRI restriction fragments were subcloned into the M13 vectors, mp18 and mp19 (Messing (1983)) and sequenced using the dideoxychain termination method of Sanger et al. (1977). The sequence obtained is shown in Fig. 1.

The polypeptide encoded in Fig. 1 that is encoded in the HCV cDNA is in the same translational frame as the N-terminal beta-galactosidase moiety to which it is 10 fused. As shown in Section IV.A., the translational open reading frame (ORF) of 5-1-1 encodes epitope(s) specifically recognized by sera from patients and chimpanzees with NANBH infections.

15 IV.A.3. <u>Isolation of Overlapping HCV cDNA to cDNA in</u> Clone 5-1-1.

Overlapping HCV cDNA to the cDNA in clone 5-1-1 was obtained by screening the same lambda-gtll library, created as described in Section IV.A.1., with a synthetic polynucleotide derived from the sequence of the HCV cDNA in clones 5-1-1, as shown in Fig. 1. The sequence of the polynucleotide used for screening was:

5'-TCC CTT GCT CGA TGT ACG GTA AGT GCT GAG AGC
25 ACT CTT CCA TCT CAT CGA ACT CTC GGT AGA GGA CTT CCC TGT
CAG GT-3'.

The lambda-gtll library was screened with this probe, using the method described in Huynh (1985). Approximately 1 in 50,000 clones hybridized with the probe. Three clones which contained cDNAs which hybridized with the synthetic probe have been numbered 81, 1-2, and 91.

IV.A.4. Nucleotide Sequences of Overlapping HCV cDNAs to cDNA in Clone 5-1-1.

The nucleotide sequences of the three cDNAs in clones 81, 1-2, and 91 were determined essentially as in 5 Section IV.A.2. The sequences of these clones relative to the HCV cDNA sequence in phage 5-1-1 is shown in Fig. 2, which shows the strand encoding the detected HCV epitope, and where the homologies in the nucleotide sequences are indicated by vertical lines between the sequences.

The sequences of the cloned HCV cDNAs are highly homologous in the overlapping regions (see Fig. 2). However, there are differences in two regions. Nucleotide 67 in clone 1-2 is a thymidine, whereas the other three clones contain a cytidine residue in this position. 15 should be noted, however, that the same amino acid is encoded when either C or T occupies this position.

The second difference is that clone 5-1-1 contains 28 base pairs which are not present in the other three clones. These base pairs occur at the start of the 20 cDNA sequence in 5-1-1, and are indicated by small letters. Based on radioimmunoassay data, which is discussed infra in Section IV.D., it is possible that an HCV epitope may be encoded in this 28 bp region.

The absence of the 28 base pairs of 5-1-1 from 25 clones 81, 1-2, and 91 may mean that the cDNA in these clones were derived from defective HCV genomes; alternatively, the 28 bp region could be a terminal artifact in clone 5-1-1.

The sequences of small letters in the nucleotide 30 sequence of clones 81 and 91 simply indicate that these sequences have not been found in other cDNAs because cDNAs overlapping these regions were not yet isolated.

A composite HCV cDNA sequence derived from overlapping cDNAs in clones 5-1-1, 81, 1-2 and 91 is shown in 35 Fig. 3. However, in this figure the unique 28 base pairs

of clone 5-1-1 are omitted. The figure also shows the sequence of the polypeptide encoded within the ORF of the composite HCV cDNA.

5 IV.A.5. <u>Isolation of Overlapping HCV cDNAs to cDNA in</u> Clone 81.

The isolation of HCV cDNA sequences upstream of, and which overlap those in clone 81 cDNA was accomplished as follows. The lambda-gtll cDNA library prepared as described in Section IV.A.1. was screened by hybridization with a synthetic polynucleotide probe which was homologous to a 5' terminal sequence of clone 81. The sequence of clone 81 is presented in Fig. 4. The sequence of the synthetic polynucleotide used for screening was:

5' CTG TCA GGT ATG ATT GCC GGC TTC CCG GAC 3'.

The methods were essentially as described in Huynh (1985), except that the library filters were given two washes

under stringent conditions, i.e., the washes were in 5 x

SSC, 0.1% SDS at 55°C for 30 minutes each. Approximately

1 in 50,000 clones hybridized with the probe. A positive recombinant phage which contained cDNA which hybridized with the sequence was isolated and purified. This phage

has been numbered clone 36.

Downstream cDNA sequences, which overlaps the carboxyl-end sequences in clone 81 cDNA were isolated using a procedure similar to that for the isolation of upstream cDNA sequences, except that a synthetic oligonucleotide probe was prepared which is homologous to a 3' terminal sequence of clone 81. The sequence of the synthetic polynucleotide used for screening was:

5' TTT GGC TAG TGG TTA GTG GGC TGG TGA CAG 3'

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A positive recombinant phage, which contained cDNA which hybridized with this latter sequence was isolated and purified, and has been numbered clone 32.

The nucleotide sequence of HCV cDNA in Clone 36.

The nucleotide sequence of the cDNA in clone 36 was determined essentially as described in Section IV.A.2.

The double-stranded sequence of this cDNA, its region of overlap with the HCV cDNA in clone 81, and the polypeptide encoded by the ORF are shown in Fig. 5.

The ORF in clone 36 is in the same translational frame as the HCV antigen encoded in clone 81. Thus, in combination, the ORFs in clones 36 and 81 encode a polypeptide that represents part of a large HCV antigen.

The sequence of this putative HCV polypeptide and the double stranded DNA sequence encoding it, which is derived from the combined ORFs of the HCV cDNAs of clones 36 and

81, is shown in Fig. 6.

The nucleotide sequence of HCV cDNA in Clone 32
was determined essentially as was that described in Section IV.A.2 for the sequence of clone 5-1-1. The sequence data indicated that the cDNA in clone 32 recombinant phage was derived from two different sources. One fragment of the cDNA was comprised of 418 nucleotides derived from the HCV genome; the other fragment was comprised of 172 nucleotides derived from the bacteriophage MS2 genome, which had been used as a carrier during the preparation of the lambda gt11 plasma cDNA library.

The sequence of the cDNA in clone 32 corresponding to that of the HCV genome is shown in Fig. 7. The region of the sequences that overlaps that of clone 81, and the polypeptide encoded by the ORF are also indicated in the figure. This sequence contains one continuous ORF

that is in the same translational frame as the HCV antigen encoded by clone 81.

IV.A.8 <u>Isolation of Overlapping HCV cDNA to cDNA in Clone</u> 5 <u>36</u>

The isolation of HCV cDNA sequences upstream of, and which overlap those in clone 36 cDNA was accomplished as described in Section IV.A.5, for those which overlap clone 81 cDNA, except that the synthetic polynucleotide

10 was based on the 5'-region of clone 36. The sequence of the synthetic polynucleotide used for screening was:

5' AAG CCA CCG TGT GCG CTA GGG CTC AAG CCC 3'

- 15 Approximately 1 in 50,000 clones hybridized with the probe. The isolated, purified clone of recombinant phage which contained cDNA which hybridized to this sequence was named clone 35.
- 20 IV.A.9 Nucleotide Sequence of HCV cDNA in Clone 35

 The nucleotide sequence of the cDNA in clone 35
 was determined essentially as described in Section IV.A.2.
 The sequence, its region of overlap with that of the cDNA in clone 36, and the putative polypeptide encoded therein,
 25 are shown in Fig. 8.

Clone 35 apparently contains a single, continuous GRF that encodes a polypeptide in the same translational frame as that encoded by clone 36, clone 81, and clone 32. Fig. 9 shows the sequence of the long continuous ORF that extends through clones 35, 36, 81, and 32, along with the putative HCV polypeptide encoded therein. This combined sequence has been confirmed using other independent cDNA clones derived from the same lambda gtll cDNA library.

IV.A.10. <u>Isolation of Overlapping HCV cDNA to cDNA in</u> Clone 35

The isolation of HCV cDNA sequences upstream of, and which overlap those in clone 35 cDNA was accomplished as described in Section IV.A.8, for those which overlap clone 36 cDNA, except that the synthetic polynucleotide was based on the 5'-region of clone 35. The sequence of the synthetic polynucleotide used for screening was:

5' CAG GAT GCT GTC TCC CGC ACT CAA CGT 3'

Approximately 1 in 50,000 clones hybridized with the probe. The isolated, purified clone of recombinant phage which contained cDNA which hybridized to this sequence was named clone 37b.

IV.A.11. Nucleotide Sequence of HCV in Clone 37b

The nucleotide sequence of the cDNA in clone 37b
was determined essentially as described in Section IV.A.2.

The sequence, its region of overlap with that of the cDNA in clone 35, and the putative polypeptide encoded therein, are shown in Fig. 10.

The 5'-terminal nucleotide of clone 35 is a T, whereas the corresponding nucleotide in clone 37b is an A.

The cDNAs from three other independent clones which were isolated during the procedure in which clone 37b was isolated, described in Section IV.A.10, have also been sequenced. The cDNAs from these clones also contain an A in this position. Thus, the 5'-terminal T in clone 35 may be an artefact of the cloning procedure. It is known that artefacts often arise at the 5'-termini of cDNA molecules.

Clone 37b apparently contains one continuous ORF which encodes a polypeptide which is a continuation of the polypeptide encoded in the ORF which extends through the overlapping clones 35, 36, 81 and 32.

IV.A.12 <u>Isolation of Overlapping HCV cDNA to cDNA in</u> Clone 32

The isolation of HCV cDNA sequences downstream of clone 32 was accomplished as follows. First, clone cla was isolated utilizing a synthetic hybridization probe which was based on the nucleotide sequence of the HCV cDNA sequence in clone 32. The method was essentially that described in Section IV.A.5, except that the sequence of the synthetic probe was:

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5' AGT GCA GTG GAT GAA CCG GCT GAT AGC CTT 3'.

Utilizing the nucleotide sequence from clone cla, another synthetic nucleotide was synthesized which had the sequence:

5' TCC TGA GGC GAC TGC ACC AGT GGA TAA GCT 3'.

Screening of the lambda gtll library using the clone cla 20 derived sequence as probe yielded approximately 1 in 50,000 positive colonies. An isolated, purified clone which hybridized with this probe was named clone 33b.

IV.A.13 Nucleotide Sequence of HCV cDNA in Clone 33b

The nucleotide sequence of the cDNA in clone 33b
was determined essentially as described in Section IV.A.2.
The sequence, its region of overlap with that of the cDNA
in clone 32, and the putative polypeptide encoded therein,
are shown in Fig. 11.

Clone 33b apparently contains one continuous ORF which is an extension of the ORFs in overlapping clones 37b, 35, 36, 81 and 32. The polypeptide encoded in clone 33b is in the same translational frame as that encoded in the extended ORF of these overlapping clones.

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IV.A:14 <u>Isolation of Overlapping HCV cDNAs to cDNA Clone</u> 37b and to cDNA in Clone 33b

In order to isolate HCV cDNAs which overlap the cDNAs in clone 37b and in clone 33b, the following synthetic oligonucleotide probes, which were derived from the cDNAs in those clones, were used to screen the lambda gt11 library, using essentially the method described in Section IV.A.3. The probes used were:

5' CAG GAT GCT GTC TCC CGC ACT CAA CGT C 3'

and

5' TCC TGA GGC GAC TGC ACC AGT GGA TAA GCT 3'

to detect colonies containing HCV cDNA sequences which overlap those in clones 37b and 33b, respectively. Approximately 1 in 50,000 colonies were detected with each probe. A clone which contained cDNA which was upstream of, and which overlapped the cDNA in clone 37b, was named clone 40b. A clone which contained cDNA which was downstream of, and which overlapped the cDNA in clone 33b was named clone 25c.

IV.A.15 Nucleotide Sequences of HCV cDNA in clone 40b and in clone 25c

The nucleotide sequences of the cDNAs in clone 40b and in clone 25c were determined essentially as described in Section IV.A.2. The sequences of 40b and 25c, their regions of overlap with the cDNAs in clones 37b and 33b, and the putative polypeptides encoded therein, are shown in Fig. 12 (clone 40b) and Fig. 13 (clone 25c).

The 5'-terminal nucleotide of clone 40b is a G.

35 However, the cDNAs from five other independent clones

which were isolated during the procedure in which clone 40b was isolated, described in Section IV.A.14, have also been sequenced. The cDNAs from these clones also contain a T in this position. Thus, the G may represent a cloning artifact (see the discussion in Section IV.A.11).

The 5'-terminus of clone 25c is ACT, but the sequence of this region in clone cla (sequence not shown), and in clone 33b is TCA. This difference may also represent a cloning artifact, as may the 28 extra 5'-terminal nucleotides in clone 5-1-1.

Clones 40b and 25c each apparently contain an ORF which is an extension of the continuous ORF in the previously sequenced clones. The nucleotide sequence of the ORF extending through clones 40b, 37b, 35, 36, 81, 32, 33b, and 25c, and the amino acid sequence of the putative polypeptide encoded therein, are shown in Fig. 14. In the figure, the potential artifacts have been omitted from the sequence, and instead, the corresponding sequences in non-5'-terminal regions of multiple overlapping clones are .

IV.A.16. Preparation of a Composite HCV cDNA from the cDNAs in Clones 36, 81, and 32

The composite HCV cDNA, C100, was constructed as follows. First the cDNAs from the clones 36, 81, and 32 were excised with EcoRI. The EcoRI fragment of cDNA from each clone was cloned individually into the EcoRI site of the vector pGEM3-blue (Promega Biotec). The resulting recombinant vectors which contained the cDNAs from clones 36, 81, and 32 were named pGEM3-blue/36, pGEM3-blue/81, and pGEM3-blue/32, respectively. The appropriately oriented recombinant of pGEM3-blue/81 was digested with NaeI and NarI, and the large (~2850bp) fragment was purified and ligated with the small (~570bp) NaeI/NarI purified restriction fragment from pGEM3-blue/36. This

composite of the cDNAs from clones 36 and 81 was used to generate another pGEM3-blue vector containing the continuous HCV ORF contained within the overlapping cDNA within these clones. This new plasmid was then digested 5 with PvuII and EcoRI to release a fragment of approximately 680bp, which was then ligated with the small (580bp) PvuII/EcoRI fragment isolated from the appropriately oriented pGEM3-blue/32 plasmid, and the composite cDNA from clones 36, 81, and 32 was ligated into the EcoRI linearized vector pSODcf1, which is described in Section IV.B.1, and which was used to express clone 5-1-1 in bacteria. Recombinants containing the ~1270bp EcoRI fragment of composite HCV cDNA (C100) were selected, and the cDNA from the plasmids was excised with EcoRI and purified.

IV.A.17. <u>Isolation and Nucleotide Sequences of HCV cDNAs</u> in Clones 14i, 11b, 7f, 7e, 8h, 33c, 14c, 8f, 33f, 33g, and 39c

The HCV cDNAs in clones 14i, 11b, 7f, 7e, 8h, 33c, 14c, 8f, 33f, 33g, and 39c were isolated by the technique of isolating overlapping cDNA fragments from the lambda gtll library of HCV cDNAs described in Section IV.A.1.. The technique used was essentially as described in Section IV.A.3., except that the probes used were designed from the nucleotide sequence of the last isolated clones from the 5' and the 3' end of the combined HCV sequences. The frequency of clones which hybridized with the probes described below was approximately 1 in 50,000 in each case.

The nucleotide sequences of the HCV cDNAs in clones 14i, 7f, 7e, 8h, 33c, 14c, 8f, 33f, 33g, and 39c were determined essentially as described in Section IV.A.2., except that the cDNA excised from these phages were substituted for the cDNA isolated from clone 5-1-1.

Clone 33c was isolated using a hybridization probe based on the sequence of nucleotides in clone 40b. The nucleotide sequence of clone 40b is presented in Fig. 12. The nucleotide sequence of the probe used to isolate 5 33c was:

5' ATC AGG ACC GGG GTG AGA ACA ATT ACC ACT 3'

The sequence of the HCV cDNA in clone 33c, and the overlap with that in clone 40b, is shown in Fig. 15, which also shows the amino acids encoded therein.

Clone 8h was isolated using a probe based on the sequence of nucleotides in clone 33c. The nucleotide sequence of the probe was

5' AGA GAC AAC CAT GAG GTC CCC GGT GTT C 3'.

The sequence of the HCV cDNA in clone 8h, and the overlap with that in clone 33c, and the amino acids encoded 20 therein, are shown in Fig. 16.

Clone 7e was isolated using a probe based on the sequence of nucleotides in clone 8h. The nucleotide sequence of the probe was

25 5' TCG GAC CTT TAC CTG GTC ACG AGG CAC 3'.

The sequence of HCV cDNA in clone 7e, the overlap with clone 8h, and the amino acids encoded therein, are shown in Fig. 17.

30 Clone 14c was isolated with a probe based on the sequence of nucleotides in clone 25c. The sequence of clone 25c is shown in Fig. 13. The probe in the isolation of clone 14c had the sequence

35 5' ACC TTC CCC ATT AAT GCC TAC ACC ACG GGC 3'.

C11000014 ----

The sequence of HCV cDNA in clone 14c, its overlap with that in clone 25c, and the amino acids encoded therein are shown in Fig. 18.

Clone 8f was isolated using a probe based on the sequence of nucleotides in clone 14c. The nucleotide sequence of the probe was

5' TCC ATC TCT CAA GGC AAC TTG CAC CGC TAA 3'.

10 The sequence of HCV cDNA in clone 8f, its overlap with that in clone 14c, and the amino acids encoded therein are shown in Fig. 19.

Clone 33f was isolated using a probe based on the nucleotide sequence present in clone 8f. The nucleotide sequence of the probe was

5' TCC ATG GCT GTC CGC TTC CAC CTC CAA AGT 3'.

The sequence of HCV cDNA in clone 33f, its overlap with 20 that in clone 8f, and the amino acids encoded therein are shown in Fig. 20.

Clone 33g was isolated using a probe based on the sequence of nucleotides in clone 33f. The nucleotide sequence of the probe was

25

5' GCG ACA ATA CGA CAA CAT CCT CTG AGC CCG 3'.

The sequence of HCV cDNA in clone 33g, its overlap with that in clone 33f, and the amino acids encoded therein are 30 shown in Fig. 21.

Clone 7f was isolated using a probe based on the sequence of nucleotides in clone 7e. The nucleotide sequence of the probe was

35 5' AGC AGA CAA GGG GCC TCC TAG GGT GCA TAA T 3'.

The sequence of HCV cDNA in clone 7f, its overlap with clone 7e, and the amino acids encoded therein are shown in Fig. 22.

Clone 11b was isolated using a probe based on 5 the sequence of clone 7f. The nucleotide sequence of the probe was

5' CAC CTA TGT TTA TAA CCA TCT CAC TCC TCT 3'.

10 The sequence of HCV cDNA in clone 11b, its overlap with clone 7f, and the amino acids encoded therein are shown in Fig. 23.

Clone 14i was isolated using a probe based on the sequence of nucleotides in clone 11b. The nucleotide sequence of the probe was

5' CTC TGT CAC CAT ATT ACA AGC GCT ATA TCA 3'.

The sequence of HCV cDNA in clone 14i, its overlap with 20 llb, and the amino acids encoded therein are shown in Fig. 24.

Clone 39c was isolated using a probe based on the sequence of nucleotides in clone 33g. The nucleotide sequence of the probe was

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5' CTC GTT GCT ACG TCA CCA CAA TTT GGT GTA 3'

The sequence of HCV cDNA in clone 39c, its overlap with clone 33g, and the amino acids encoded therein are shown in Fig. 25.

IV.A.18. The Composite HCV cDNA Sequence Derived from Isolated Clones Containing HCV cDNA

The HCV cDNA sequences in the isolated clones described supra have been aligned to create a composite

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HCV cDNA sequence. The isolated clones, aligned in the 5' to 3' direction are: 14i, 7f, 7e, 8h, 33c, 40b, 37b, 35, 36, 81, 32, 33b, 25c, 14c, 8f, 33f, 33g, and 39c.

A composite HCV cDNA sequence derived from the isolated clones, and the amino acids encoded therein, is shown in Fig. 26.

In creating the composite sequence the following sequence heterogeneities have been considered. Clone 33c contains an HCV cDNA of 800 base pairs, which overlaps the cDNAs in clones 40b and 37c. In clone 33c, as well as in 5 other overlapping clones, nucleotide #789 is a G. However, in clone 37b (see Section IV.A.11), the corresponding nucleotide is an A. This sequence difference creates an apparent heterogeneity in the amino acids encoded therein, which would be either CYS or TYR, for G or A, respectively. This heterogeneity may have important ramifications in terms of protein folding.

Nucleotide residue #2 in clone 8h HCV cDNA is a T. However, as shown infra, the corresponding residue in clone 7e is an A; moreover, an A in this position is also found in 3 other isolated overlapping clones. Thus, the T residue in clone 8h may represent a cloning artifact. Therefore, in Fig. 26, the residue in this position is designated as an A.

25 The 3'-terminal nucleotide in clone 8f HCV cDNA is a G. However, the corresponding residue in clone 33f, and in 2 other overlapping clones is a T. Therefore, in Fig. 26, the residue in this position is designated as a T.

30 The 3'-terminal sequence in clone 33f HCV cDNA is TTGC. However, the corresponding sequence in clone 33g and in 2 other overlapping clones is ATTC. Therefore, in Fig. 26, the corresponding region is represented as ATTC.

Nucleotide residue #4 in clone 33g HCV cDNA is a 35 T. However, in clone 33f and in 2 other overlapping

clones the corresponding residue is an A. Therefore, in Fig. 26, the corresponding residue is designated as an A.

The 3'-terminus of clone 14i is an AA, whereas the corresponding dinucleotide in clone 11b, and in three other clones, is TA. Therefore, in Fig. 26, the TA residue is depicted.

The resolution of other sequence heterogeneities is discussed supra.

An examination of the composite HCV cDNA

10 indicates that it contains one large ORF. This suggests
that the viral genome is translated into a large
polypeptide which is processed concomitant with, or
subsequent to translation.

15 IV.A.19. <u>Isolation and Nucleotide Sequences of HCV cDNAs</u> in Clones 12f, 35f, 19g, 26g, and 15e

The HCV cDNAs in clones 12f, 35f, 19g, 26g, and 15e were isolated essentially by the technique described in Section IV.A.17, except that the probes were as indicated below. The frequency of clones which hybridized with the probes was approximately 1 in 50,000 in each case. The nucleotide sequences of the HCV cDNAs in these clones were determined essentially as described in Section IV.A.2., except that the cDNA from the indicated clones were substituted for the cDNA isolated from clone 5-1-1.

The isolation of clone 12f, which contains cDNA upstream of the HCV cDNA in Fig. 26, was accomplished using a hybridization probe based on the sequence of nucleotides in clone 14i. The nucleotide sequence of the 30 probe was

5' TGC TTG TGG ATG ATG CTA CTC ATA TCC CAA 3'.

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The HCV cDNA sequence of clone 12f, its Overlap with clone 14i, and the amino acids encoded therein are shown in Fig. 27.

The isolation of clone 35f, which contains cDNA downstream of the HCV cDNA in Fig. 26, was accomplished using a hybridization probe based on the sequence of nucleotides in clone 39c. The nucleotide sequence of the probe was

5' AGC AGC GGC GTC AAA AGT GAA GGC TAA CTT 3'.

The sequence of clone 35f, its overlap with the sequence in clone 39c, and the amino acids encoded therein are shown in Fig. 28.

15 The isolation of clone 19g was accomplished using a hybridization probe based on the 3' sequence of clone 35f. The nucleotide sequence of the probe was

5' TTC TCG TAT GAT ACC CGC TGC TTT GAC TCC 3'.

The HCV cDNA sequence of clone 19g, its overlap with the sequence in clone 35f, and the amino acids encoded therein are shown in Fig. 29.

The isolation of clone 26g was accomplished 25 using a hybridization probe based on the 3' sequence of clone 19g. The nucleotide sequence of the probe was

- 5' TGT GTG GCG ACG ACT TAG TCG TTA TCT GTG 3'.
- 30 The HCV cDNA sequence of clone 26g, its overlap with the sequence in clone 19g, and the amino acids encoded therein are shown in Fig. 30.

Clone 15e was isolated using a hybridization probe based on the 3' sequence of clone 26 g. The nucleotide sequence of the probe was

5' CAC ACT CCA GTC AAT TCC TGG CTA GGC AAC 3'.

The HCV cDNA sequence of clone 15e, its overlap with the sequence in clone 26g, and the amino acids encoded therein are shown in Fig. 31.

The clones described in this Section have been deposited with the ATCC under the terms and conditions described in Section II.A., and have been assigned the following Accession Numbers.

10

	lambd	a-gt11	ATCC No.	De	posit	Date
	clone	12f	40514	10	Nov.	1988
	clone	35f	40511	10	Nov.	1988
	clone	15e	40513	10	Nov.	1988
15	clone	K9-1	40512	10	Nov.	1988

The HCV cDNA sequences in the isolated clones described supra. have been aligned to create a composite HCV cDNA sequence. The isolated clones, aligned in the 5' to 3' direction are: 12f, 14i, 7f, 7e, 8h, 33c, 40b, 37b, 35, 36, 81, 32, 33b, 25c, 14c, 8f 33f, 33g, 39c, 35f, 19g, 26g, and 15e.

A composite HCV cDNA sequence derived from the isolated clones, and the amino acids encoded therein, is shown in Fig. 32.

IV.A.20. <u>Alternative Method of Isolating cDNA Sequences</u> Upstream of the HCV cDNA Sequence in Clone 12f

Based on the most 5' HCV sequence in Fig. 32,
30 which is derived from the HCV cDNA in clone 12f, small
synthetic oligonucleotide primers of reverse transcriptase
are synthesized and used to bind to the corresponding
sequence in HCV genomic RNA, to prime reverse
transcription of the upstream sequences. The primer

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sequences are proximal to the known 5'-terminal sequence of clone 12f, but sufficiently downstream to allow the design of probe sequences upstream of the primer sequences. Known standard methods of priming and cloning are used. The resulting cDNA libraries are screened with sequences upstream of the priming sites (as deduced from the elucidated sequence in clone 12f). The HCV genomic RNA is obtained from either plasma or liver samples from chimpanzees with NANBH, or from analogous samples from humans with NANBH.

IV.A.21. Alternative Method Utilizing Tailing to Isolate Sequences from the 5'-Terminal Region of the HCV Genome In order to isolate the extreme 5'-terminal sequences of the HCV RNA genome, the cDNA product of the first round of reverse transcription, which is duplexed with the template RNA, is tailed with oligo C. This is accomplished by incubating the product with terminal transferase in the presence of CTP. The second round of cDNA synthesis, which yields the complement of the first strand of cDNA, is accomplished utilizing oligo G as a primer for the reverse transcriptase reaction. The sources of genomic HCV RNA are as described in Section IV.A.20. The methods for tailing with terminal transferase, and for the reverse transcriptase reactions are as in Maniatis et al. (1982). The cDNA products are

IV.A.22. Alternative Method Utilizing Tailing to Isolate

Sequences from the 3'-Terminal Region of the HCV Genome

This method is based on previously used methods for cloning cDNAs of Flavivirus RNA. In this method, the RNA is subjected to denaturing conditions to remove secondary structures at the 3'-terminus, and is then tailed with Poly A polymerase using rATP as a substrate.

then cloned, screened, and sequenced.

Reverse transcription of the poly A tailed RNA is catalyzed by reverse transcriptase, utilizing oligo dT as a primer. The second strands of cDNA are synthesized, the cDNA products are cloned, screened, and sequenced.

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IV.A.23 <u>Creation of Lambda-qt11 HCV cDNA Libraries</u> Containing Larger cDNA Inserts

The method used to create and screen the Lambda gt11 libraries are essentially as described in Section

10 IV.A.1., except that the library is generated from a pool of larger size cDNAs eluted from the Sepharose CL-4B column.

IV.A.24. <u>Creation of HCV cDNA Libraries Using Synthetic</u> 15 Oligomers as Primers

New HCV cDNA libraries have been prepared from the RNA derived from the infectious chimpanzee plasma pool described in Section IV.A.1., and from the poly A⁺ RNA fraction derived from the liver of this infected animal.

20 The cDNA was constructed essentially as described by Gubler and Hoffman (1983), except that the primers for the first cDNA strand synthesis were two synthetic oligomers based on the sequence of the HCV genome described supra. Primers based on the sequence of clone 11 b and 7e were,

5' CTG GCT TGA AGA ATC 3'

and

30

25 respectively,

5' AGT TAG GCT GGT GAT TAT GC 3'.

The resulting cDNAs were cloned into lambda bacteriophage vectors, and screened with various other synthetic

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oligomers, whose sequences were based on the HCV sequence in Fig. 32.

IV.B. Expression of Polypeptides Encoded Within HCV cDNAs and Identification of the Expressed Products as HCV Induced Antigens.

IV.B.1. Expression of the Polypeptide Encoded in Clone 5-1-1.

10 The HCV polypeptide encoded within clone 5-1-1 (see Section IV.A.2., supra) was expressed as a fusion polypeptide with superoxide dismutase (SOD). This was accomplished by subcloning the clone 5-1-1 cDNA insert into the expression vector pSODcfl (Steimer et al. (1986)) as follows.

First, DNA isolated from pSODcfl was treated with BamHI and EcoRI, and the following linker was ligated into the linear DNA created by the restriction enzymes:

20 5' GAT CCT GGA ATT CTG ATA A 3'
3' GA CCT TAA GAC TAT TTT AA 5'

After cloning, the plasmid containing the insert was

isolated.

Plasmid containing the insert was restricted with EcoRI. The HCV cDNA insert in clone 5-1-1 was excised with EcoRI, and ligated into this EcoRI linearized plasmid DNA. The DNA mixture was used to transform E.

coli strain D1210 (Sadler et al. (1980)). Recombinants with the 5-1-1 cDNA in the correct orientation for

expression of the ORF shown in Fig. 1 were identified by restriction mapping and nucleotide sequencing.

Recombinant bacteria from one clone were induced to express the SOD-NANB $_{\rm 5-1-1}$ polypeptide by growing the bacteria in the presence of IPTG.

shown in Fig. 4.

IV.B.2. Expression of the Polypeptide Encoded in Clone 81.

The HCV cDNA contained within clone 81 was expressed as a SOD-NANB₈₁ fusion polypeptide. The method 5 for preparing the vector encoding this fusion polypeptide was analogous to that used for the creation of the vector encoding SOD-NANB₅₋₁₋₁, except that the source of the HCV cDNA was clone 81, which was isolated as described in Section IV.A.3, and for which the cDNA sequence was determined as described in Section IV.A.4. The nucleotide sequence of the HCV cDNA in clone 81, and the putative amino acid sequence of the polypeptide encoded therein are

The HCV cDNA insert in clone 81 was excised with 15 EcoRI, and ligated into the pSODcfl which contained the linker (see IV.B.1.) and which was linearized by treatment with EcoRI. The DNA mixture was used to transform E. colistrain D1210. Recombinants with the clone 81 HCV cDNA in the correct orientation for expression of the ORF shown in Fig. 4 were identified by restriction mapping and nucleotide sequencing.

Recombinant bacteria from one clone were induced to express the SOD-NANB₈₁ polypeptide by growing the bacteria in the presence of IPTG.

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IV.B.3. <u>Identification of the Polypeptide Encoded Within</u> Clone 5-1-1 as an HCV and NANBH Associated Antigen.

The polypeptide encoded within the HCV cDNA of clone 5-1-1 was identified as a NANBH associated antigen by demonstrating that sera of chimpanzees and humans infected with NANBH reacted immunologically with the fusion polypeptide, SOD-NANB₅₋₁₋₁, which is comprised of superoxide dismutase at its N-terminus and the in-frame 5-1-1 antigen at its C-terminus. This was accomplished by "Western" blotting (Towbin et al. (1979)) as follows.

with an expression vector encoding the SOD-NANB₅₋₁₋₁ polypeptide, described in Section IV.B.I., was induced to express the fusion polypeptide by growth in the presence of IPTG. Total bacterial lysate was subjected to electrophoresis through polyacrylamide gels in the presence of SDS according to Laemmli (1970). The separated polypeptides were transferred onto nitrocellulose filters (Towbin et al. (1979)). The filters were then cut into thin strips, and the strips were incubated individually with the different chimpanzee and human sera. Bound antibodies were detected by further incubation with ¹²⁵I-labeled sheep anti-human Ig, as described in Section IV.A.1.

for the Western blots and the results, shown in the photograph of the autoradiographed strips, are presented in Fig. 33. Nitrocellulose strips containing polypeptides were incubated with sera derived from chimpanzees at different times during acute NANBH (Hutchinson strain) infections (lanes 1-16), hepatitis A infections (lanes 17-24, and 26-33), and hepatitis B infections (lanes 34-44). Lanes 25 and 45 show positive controls in which the immunoblots were incubated with serum from the patient used to identify the recombinant clone 5-1-1 in the original screening of the lambda-gtll cDNA library (see Section IV.A.1.).

The band visible in the control lanes, 25 and 45, in Fig. 23 reflects the binding of antibodies to the NANB₅₋₁₋₁ moiety of the SOD fusion polypeptide. These antibodies do not exhibit binding to SOD alone, since this has also been included as a negative control in these samples, and would have appeared as a band migrating significantly faster than the SOD-NANB₅₋₁₋₁ fusion polypeptide.

Lanes 1-16 of Fig. 33 show the binding of antibodies in sera samples of 4 chimpanzees; the sera were
obtained just prior to infection with NANBH, and
sequentially during acute infection. As seen from the
figure, whereas antibodies which reacted immunologically
with the SOD-NANB₅₋₁₋₁ polypeptide were absent in sera
samples obtained before administration of infectious HCV
inoculum and during the early acute phase of infection,
all 4 animals eventually induced circulating antibodies to
this polypeptide during the late part of, or following the
acute phase. Additional bands observed on the immunoblots
in the cases of chimps numbers 3 and 4 were due to
background binding to host bacterial proteins.

In contrast to the results obtained with sera

15 from chimps infected with NANBH, the development of antibodies to the NANB₅₋₁₋₁ moiety of the fusion polypeptide
was not observed in 4 chimpanzees infected with HAV or 3
chimpanzees infected with HBV. The only binding in these
cases was background binding to the host bacterial

20 proteins, which also occurred in the HCV infected samples.

The characterization of the human sera used for the Western blots, and the results, which are shown in the photograph of the autoradiographed strips, are presented in Fig. 34. Nitrocellulose strips containing polypeptides were incubated with sera derived from humans at different times during infections with NANBH (lanes 1-21), HAV (lanes 33-40), and HBV (lanes 41-49). Lanes 25 and 50 show positive controls in which the immunoblots were incubated with serum from patient used in the original screening of the lambda-gtll library, described supra. Lanes 22-24 and 26-32 show "non-infected" controls in which the sera was from "normal" blood donors.

As seen in Fig. 34, sera from nine NANBH patients, including the serum used for screening the lambda-gtll library, contained antibodies to the NANB5-1-1

moiety of the fusion polypeptide. Sera from three patients with NANBH did not contain these antibodies. It is possible that the $anti-NANB_{5-1-1}$ antibodies will develop at a future date in these patients. It is also 5 possible that this lack of reaction resulted from a different NANBV agent being causative of the disease in the individuals from which the non-responding serum was taken.

Fig. 34 also shows that sera from many patients infected with HAV and HBV did not contain anti-NANB $_{5-1-1}$ 10 antibodies, and that these antibodies were also not present in the sera from "normal" controls. Although one HAV patient (lane 36) appears to contain anti-NANB₅₋₁₋₁ antibodies, it is possible that this patient had been previously infected with HCV, since the incidence of NANBH 15 is very high and since it is often subclinical.

These serological studies indicate that the cDNA in clone 5-1-1 encodes epitopes which are recognized specifically by sera from patients and animals infected with BB-NANBV. In addition, the cDNA does not appear to 20 be derived from the primate genome. A hybridization probe made from clone 5-1-1 or from clone 81 did not hybridize to "Southern" blots of control human and chimpanzee genomic DNA from uninfected individuals under conditions where unique, single-copy genes are detectable. 25 probes also did not hybridize to Southern blots of control bovine genomic DNA.

Expression of the Polypeptide Encoded in a Composite of the HCV cDNAs in Clones 36, 81 and 32

The HCV polypeptide which is encoded in the ORF which extends through clones 36, 81 and 32 was expressed as a fusion polypeptide with SOD. This was accomplished by inserting the composite cDNA, C100, into an expression cassette which contains the human superoxide dismutase 35 gene, inserting the expression cassette into a yeast

expression vector, and expressing the polypeptide in yeast.

An expression cassette containing the composite C100 cDNA derived from clones 36, 81, and 32, was constructed by inserting the ~1270bp EcoRI fragment into the EcoRI site of the vector pS3-56 (also called pS356), yielding the plasmid pS3-56_{C100}. The construction of C100 is described in Section IV.A.16, supra.

The vector pS3-56, which is a pBR322 derivative,

10 contains an expression cassette which is comprised of the

ADH2/GAPDH hybrid yeast promoter upstream of the human

superoxide dismutase gene, and a downstream GAPDH

transcription terminator. A similar cassette, which

contains these control elements and the superoxide

15 dismutase gene has been described in Cousens et al.

(1987), and in copending application EPO 196,056,

published October 1, 1986, which is commonly owned by the

herein assignee. The cassette in pS3-56, however, differs

from that in Cousens et al. (1987) in that the

20 heterologous proinsulin gene and the immunoglobulin hinge

are deleted, and in that the gln₁₅₄ of the superoxide

dismutase is followed by an adaptor sequence which

25 5'-AAT TTG GGA ATT CCA TAA TGA G -3'
AC CCT TAA GGT ATT ACT CAG CT

The EcoRI site allows the insertion of heterologous sequences which, when expressed from a vector containing the cassette, yield polypeptides which are fused to superoxide dismutase via an oligopeptide linker containing the amino acid sequence:

contains an EcoRI site. The sequence of the adaptor is:

-asn-leu-gly-ile-arg-.

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A sample of pS356 has been deposited on 29 April 1988 under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville, Maryland 20853, and has been assigned Ac-5 cession No. 67683. The terms and conditions for availability and access to the deposit, and for maintenance of the deposit are the same as those specified in Section II.A., for strains containing NANBV-cDNAs. This deposit is intended for convenience only, and is not required to 10 practice the present invention in view of the description here. The deposited material is hereby incorporated herein by reference.

After recombinants containing the C100 cDNA insert in the correct orientation were isolated, the 15 expression cassette containing the C100 cDNA was excised from $ps3-56_{cl00}$ with BamHI, and a fragment of ~3400bp which contains the cassette was isolated and purified. This fragment was then inserted into the BamHI site of the yeast vector pAB24.

Plasmid pAB24, the significant features of which are shown in Fig. 35, is a yeast shuttle vector which contains the complete 2 micron sequence for replication [Broach (1981)] and pBR322 sequences. It also contains the yeast URA3 gene derived from plasmid YEp24 [Botstein 25 et al. (1979)], and the yeast Leu^{2d} gene derived from plasmid pC1/1. EPO Pub. No. 116,201. Plasmid pAB24 was constructed by digesting YEp24 with EcoRI and religating the vector to remove the partial 2 micron sequences. The resulting plasmid, YEP24deltaRI, was linearized by 30 digestion with ClaI and ligated with the complete 2 micron plasmid which had been linearized with ClaI. The resulting plasmid, pCBou, was then digested with XbaI and the 8605 bp vector fragment was gel isolated. This isolated XbaI fragment was ligated with a 4460 bp XbaI fragment containing the LEU^{2d} gene isolated from pC1/1;

the orientation of the LEU^{2d} gene is in the same direction as the URA3 gene. Insertion of the expression was in the unique BamHI site of the pBR322 sequence, thus interrupting the gene for bacterial resistance to tetracycline.

The recombinant plasmid which contained the SOD-C100 expression cassette, pAB24C100-3, was transformed into yeast strain JSC 308, as well as into other yeast strains. The cells were transformed as described by 10 Hinnen et al. (1978), and plated onto ura-selective plates. Single colonies were inoculated into leuselective media and grown to saturation. The culture was induced to express the SOD-C100 polypeptide (called C100-3) by growth in YEP containing 1% glucose.

Strain JSC 308 is of the genotype MAT 0, leu2, ura3(del) DM15 (GAP/ADR1) integrated at the ADR1 locus. In JSC 308, over-expression of the positive activator gene product, ADR1, results in hyperderepression (relative to an ADR1 wild type control) and significantly higher yields 20 of expressed heterologous proteins when such proteins are synthesized via an ADH2 UAS regulatory system. construction of the yeast strain JSC 308 is disclosed in copending application, U.S. Serial No. (Attorney Docket No. 2300-0229), filed concurrently herewith, and which is hereby incorporated herein by reference. A sample of JSC 308 has been deposited on 5 May 1988 with the ATCC under the conditions of the Budapest Treaty, and has been assigned Accession No. 20879. The terms and conditions for availability and access to the deposit, and for 30 maintenance of the deposit are the same as those specified in Section II.A., for strains containing HCV cDNAs.

The complete C100-3 fusion polypeptide encoded in pAB24C100-3 should contain 154 amino acids of human SOD at the amino-terminus, 5 amino acid residues derived from the synthetic adaptor containing the EcoRI site, 363 amino

acid residues derived from C100 cDNA, and 5 carboxyterminal amino acids derived from the MS2 nucleotide
sequence adjoining the HCV cDNA sequence in clone 32.
(See Section IV.A.7.) The putative amino acid sequence of
the carboxy-terminus of this polypeptide, beginning at the
penultimate Ala residue of SOD, is shown in Fig. 36; also
shown is the nucleotide sequence encoding this portion of
the polypeptide.

10 IV.B.5. <u>Identification of the Polypeptide Encoded within</u> C100 as an NANBH Associated Antigen

The C100-3 fusion polypeptide expressed from plasmid pAB24C100-3 in yeast strain JSC 308 was characterized with respect to size, and the polypeptide encoded within C100 was identified as an NANBH-associated antigen by its immunological reactivity with serum from a human with chronic NANBH.

The C100-3 polypeptide, which was expressed as described in Section IV.B.4., was analyzed as follows. Yeast JSC 308 cells were transformed with pAB24, or with 20 pAB24C100-3, and were induced to express the heterologous The induced yeast cells in plasmid encoded polypeptide. 1 ml of culture (OD $_{650\ nm}$ ~20) were pelleted by centrifugation at 10,000 rpm for 1 minute, and were lysed by vortexing them vigorously (10 x 1 min) with 2 volumes 25 of solution and 1 volume of glass beads (0.2 millimicron diameter). The solution contained 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1mM phenylmethylsulphonyl fluoride (PMSF), and 1 microgram/ml pepstatin. Insoluble material in the lysate, which includes the C100-3 polypeptide, was col-30 lected by centrifugation (10,000 rpm for 5 minutes), and was dissolved by boiling for 5 minutes in Laemmli SDS sample buffer. [See Laemmli (1970)]. An amount of polypeptides equivalent to that in 0.3 ml of the induced yeast culture was subjected to electrophoresis through 10% 35

polyacrylamide gels in the presence of SDS according to Laemmli (1970). Protein standards were co-electrophoresed on the gels. Gels containing the expressed polypeptides were either stained with Coomassie brilliant blue, or were subjected to "Western" blotting as described in Section IV.B.2., using serum from a patient with chronic NANBH to determine the immunological reactivity of the polypeptides expressed from pAB24 and from pAB24C100-3.

The results are shown in Fig. 37. In Fig. 37A

10 the polypeptides were stained with Coomassie brilliant
blue. The insoluble polypeptide(s) from JSC 308 transformed with pAB24 and from two different colonies of JSC
transformed with pAB24C100-3 are shown in lane 1 (pAB24),
and lanes 2 and 3, respectively. A comparison of lanes 2

15 and 3 with lane 1 shows the induced expression of a
polypeptide corresponding to a molecular weight of ~54,000
daltons from JSC 308 transformed with pAB24C100-3, which
is not induced in JSC 308 transformed with pAB24. This
polypeptide is indicated by the arrow.

20 Fig. 37B shows the results of the Western blots of the insoluble polypeptides expressed in JSC 308 transformed with pAB24 (lane 1), or with pAB24C100-3 (lane 2). The polypeptides expressed from pAB24 were not immunologically reactive with serum from a human with NANBH.

25 However, as indicated by the arrow, JSC 308 transformed with pAB24C100-3 expressed a polypeptide of ~54,000 dalton molecular weight which did react immunologically with the human NANBH serum. The other immunologically reactive polypeptides in lane 2 may be degradation and/or aggregation products of this ~54,000 dalton polypeptide.

IV.B.6. <u>Purification of Fusion Polypeptide C100-3</u>

The fusion polypeptide, C100-3, comprised of SOD at the N-terminus and in-frame C100 HCV-polypeptide at the C-terminus was purified by differential extraction of the

insoluble fraction of the extracted host yeast cells in which the polypeptide was expressed.

The fusion polypeptide, C100-3, was expressed in yeast strain JSC 308 transformed with pAB24C100-3, as described in Section IV.B.4. The yeast cells were then lysed by homogenization, the insoluble material in the lysate was extracted at pH 12.0, and C100-3 in the remaining insoluble fraction was solubilized in buffer containing SDS.

The yeast lysate was prepared essentially according to Nagahuma et al. (1984). A yeast cell suspension was prepared which was 33% cells (v/v) suspended in a solution (Buffer A) containing 20 mM Tris HCl, pH 8.0, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride (PMSF). An aliquot of the suspension (15 ml) was mixed with an equal volume of glass beads (0.45-0.50 mm diameter), and the mixture was vortexed at top speed on a Super Mixer (Lab Line Instruments, Inc.) for 8 min. The

homogenate and glass beads were separated, and the glass

20 beads were washed 3 times with the same volume of Buffer A
as the original packed cells. After combining the washes
and homogenate, the insoluble material in the lysate was
obtained by centrifuging the homogenate at 7,000 x g for
15 minutes at 4°C, resuspending the pellets in Buffer A
25 equal to twice the volume of original packed cells, and
re-pelleting the material by centrifugation at 7,000 x g
for 15 min. This washing procedure was repeated 3 times.

The insoluble material from the lysate was extracted at pH 12.0 as follows. The pellet was suspended in buffer containing 0.5 M NaCl, 1 mM EDTA, where the suspending volume was equal to 1.8 times the of the original packed cells. The pH of the suspension was adjusted by adding 0.2 volumes of 0.4 M Na phosphate buffer, pH 12.0. After mixing, the suspension was centrifuged at 7,000 x g for 15 min at 4°C, and the super-

natant removed. The extraction was repeated 2 times. The extracted pellets were washed by suspending them in 0.5 M NaCl, 1 mM EDTA, using a suspension volume equal to two volumes of the original packed cells, followed by centrifugation at 7,000 x g for 15 min at 4°C.

The C100-3 polypeptide in the extracted pellet was solubilized by treatment with SDS. The pellets were suspended in Buffer A equal to 0.9 volumes of the original packed cell volume, and 0.1 volumes of 2% SDS was added.

10 After the suspension was mixed, it was centrifuged at 7,000 x g for 15 min at 4°C. The resulting pellet was extracted 3 more times with SDS. The resulting supernatants, which contained C100-3 were pooled.

This procedure purifies C100-3 more than 10-fold from the insoluble fraction of the yeast homogenate, and the recovery of the polypeptide is greater than 50%.,

The purified preparation of fusion polypeptide was analyzed by polyacrylamide gel electrophoresis according to Laemmli (1970). Based upon this analysis, the polypeptide was greater than 80% pure, and had an apparent molecular weight of ~54,000 daltons.

IV.C. <u>Identification of RNA in Infected Individuals Which</u> Hybridizes to HCV cDNA.

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IV.C.1. <u>Identification of RNA in the Liver of a Chimpanzee With NANBH Which Hybridizes to HCV cDNA.</u>

RNA from the liver of a chimpanzee which had NANBH was shown to contain a species of RNA which hybridized to the HCV cDNA contained within clone 81 by Northern blotting, as follows.

RNA was isolated from a liver biopsy of the chimpanzee from which the high titer plasma was derived (see Section TV.A.1.) using techniques described in Maniatis et al. (1982) for the isolation of total RNA from

mammalian cells, and for its separation into poly \boldsymbol{A}^{+} and poly A fractions. These RNA fractions were subjected to electrophoresis on a formaldehyde/agarose gel (1% w/v), and transferred to nitrocellulose. (Maniatis et al. 5 (1982)). The nitrocellulose filters were hybridized with radiolabeled HCV cDNA from clone 81 (see Fig. 4 for the nucleotide sequence of the insert.) To prepare the radiolabeled probe, the HCV cDNA insert isolated from clone 81 was radiolabeled with 32p by nick translation 10 using DNA Polymerase I (Maniatis et al. (1982)). Hybridization was for 18 hours at 42°C in a solution containing 10% (w/v) Dextran sulphate, 50% (w/v) deionized formamide, 750 mM NaCl, 75 mM Na citrate, 20 mM Na, HPO,, pH 6.5, 0.1% SDS, 0.02% (w/v) bovine serum albumin (BSA), 15 0.02% (w/v) Ficoll-400, 0.02% (w/v) polyvinylpyrrolidone, 100 micrograms/ml salmon sperm DNA which had been sheared by sonication and denatured, and 106 CPM/ml of the nicktranslated cDNA probe.

An autoradiograph of the probed filter is shown
in Fig. 38. Lane 1 contains 32P-labeled restriction fragment markers. Lanes 2-4 contain chimpanzee liver RNA as
follows: lane 2 contains 30 micrograms of total RNA; lane
3 contains 30 micrograms of poly A- RNA; and lane 4
contains 20 micrograms of poly A+ RNA. As shown in Fig.
38, the liver of the chimpanzee with NANBH contains a
heterogeneous population of related poly A+ RNA molecules
which hybridizes to the HCV cDNA probe, and which appears
to be from about 5000 nucleotides to about 11,000
nucleotides in size. This RNA, which hybridizes to the
HCV cDNA, could represent viral genomes and/or specific

The experiment described in Section IV.C.2., infra, is consistent with the suggestion that HCV contains an RNA genome.

transcripts of the viral genome.

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IV.C.2. <u>Identification of HCV Derived RNA in Serum from Infected Individuals</u>.

Nucleic acids were extracted from particles isolated from high titer chimpanzee NANBH plasma as 5 described in Section IV.A.1.. Aliquots (equivalent to 1 ml of original plasma) of the isolated nucleic acids were resuspended in 20 microliters 50 mM Hepes, pH 7.5, 1 mm EDTA and 16 micrograms/ml yeast soluble RNA. The samples were denatured by boiling for 5 minutes followed by im-10 mediate freezing, and were treated with RNase A (5 microliters containing 0.1 mg/ml RNase A in 25 mM EDTA, 40 mM Hepes, pH 7.5) or with DNase I (5 microliters containing 1 unit DNase I in 10 mM MgCl2, 25 mM Hepes, pH 7.5); control samples were incubated without enzyme. Following incuba-15 tion, 230 microliters of ice-cold 2XSSC containing 2 micrograms/ml yeast soluble RNA was added, and the samples were filtered on a nitrocellulose filter. The filters were hybridized with a cDNA probe from clone 81, which had been ³²P-labeled by nick-translation. Fig. 39 shows an 20 autoradiograph of the filter. Hybridization signals were detected in the DNase treated and control samples (lanes 2 and 1, respectively), but were not detected in the RNase treated sample (lane 3). Thus, since RNase A treatment destroyed the nucleic acids isolated from the particles, 25 and DNase I treatment had no effect, the evidence strongly suggests that the HCV genome is composed of RNA.

IV.C.3. <u>Detection of Amplified HCV Nucleic Acid Sequences</u> <u>derived from HCV Nucleic Acid Sequences in Liver and</u> <u>Plasma Specimens from Chimpanzees with NANBH</u>

HCV nucleic acids present in liver and plasma of chimpanzees with NANBH, and in control chimpanzees, were amplified using essentially the polymerase chain reaction (PCR) technique described by Saiki et al. (1986). The primer oligonucleotides were derived from the HCV cDNA

sequences in clone 81, or clones 36 and 37. The amplified sequences were detected by gel electrophoresis and Southern blotting, using as probes the appropriate cDNA oligomer with a sequence from the region between, but not including, the two primers.

Samples of RNA containing HCV sequences to be examined by the amplification system were isolated from liver biopsies of three chimpanzees with NANBH, and from two control chimpanzees. The isolation of the RNA fraction was by the guanidinium thiocyanate procedure described in Section IV.C.1.

Samples of RNA which were to be examined by the amplification system were also isolated from the plasmas of two chimpanzees with NANBH, and from one control chimpanzee, as well as from a pool of plasmas from control chimpanzees. One infected chimpanzee had a CID/ml equal to or greater than 10⁶, and the other infected chimpanzee had a CID/ml equal to or greater than 10⁵.

The nucleic acids were extracted from the plasma 20 as follows. Either 0.1 ml or 0.01 ml of plasma was diluted to a final volume of 1.0 ml, with a TENB/ proteinase K/SDS solution (0.05 M Tris-HCL, pH 8.0, 0.001 M EDTA, 0.1 M NaCl, 1 mg/ml Proteinase K, and 0.5% SDS) containing 10 micrograms/ml polyadenylic acid, and 25 incubated at 37°C for 60 minutes. After this proteinase K digestion, the resultant plasma fractions were deproteinized by extraction with TE (10.0 mM Tris-HCl, pH 8.0, 1 mM EDTA) saturated phenol. The phenol phase was separated by centrifugation, and was reextracted with TENB 30 containing 0.1% SDS. The resulting aqueous phases from each extraction were pooled, and extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol [1:1(99:2)], and then twice with an equal volume of a 99:1 mixture of chloroform/isoamyl alcohol. Following phase 35 separation by centrifugation, the aqueous phase was

brought to a final concentration of 0.2 M Na Acetate, and the nucleic acids were precipitated by the addition of two volumes of ethanol. The precipitated nucleic acids were recovered by ultracentrifugation in a SW 41 rotor at 38 K, for 60 minutes at 4°C.

In addition to the above, the high titer chimpanzee plasma and the pooled control plasma alternatively were extracted with 50 micrograms of poly A carrier by the procedure of Chomcyzski and Sacchi (1987).

This procedure uses an acid guanidinium thiocyanate extraction. RNA was recovered by centrifugation at 10,000 RPM for 10 minutes at 4°C in an Eppendorf microfuge.

On two occasions, prior to the synthesis of cDNA in the PCR reaction, the nucleic acids extracted from plasma by the proteinase K/SDS/phenol method were further purified by binding to and elution from S and S Elutip-R Columns. The procedure followed was according to the manufacturer's directions.

The cDNA used as a template for the PCR reaction 20 was derived from the nucleic acids (either total nucleic acids or RNA) prepared as described above. Following ethanol precipitation, the precipitated nucleic acids were dried, and resuspended in DEPC treated distilled water. Secondary structures in the nucleic acids were disrupted 25 by heating at 65°C for 10 minutes, and the samples were immediately cooled on ice. cDNA was synthesized using 1 to 3 micrograms of total chimpanzee RNA from liver, or from nucleic acids (or RNA) extracted from 10 to 100 microliters of plasma. The synthesis utilized reverse 30 transcriptase, and was in a 25 microliter reaction, using the protocol specified by the manufacturer, BRL. The primers for cDNA synthesis were those also utilized in the PCR reaction, described below. All reaction mixtures for cDNA synthesis contained 23 units of the RNAase inhibitor, 35 RNASIN (Fisher/Promega). Following cDNA synthesis, the

reaction mixtures were diluted with water, boiled for 10 minutes, and quickly chilled on ice.

The PCR reactions were performed essentially according to the manufacturer's directions (Cetus-Perkin-Elmer), except for the addition of 1 microgram of RNase A. The reactions were carried out in a final volume of 100 microliters. The PCR was performed for 35 cycles, utilizing a regimen of 37°C, 72°C, and 94°C.

The primers for cDNA synthesis and for the PCR

reactions were derived from the HCV cDNA sequences in
either clone 81, clone 36, or clone 37b. (The HCV cDNA
sequences of clones 81, 36, and 37b are shown in Figs. 4,
5, and 10, respectively.) The sequences of the two 16-mer
primers derived from clone 81 were:

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5' CAA TCA TAC CTG ACA G 3' and

5' GAT AAC CTC TGC CTG A 3'.

20 The sequence of the primer from clone 36 was:

5' GCA TGT CAT GAT GTA T 3'.

The sequence of the primer from clone 37b was:

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5' ACA ATA CGT GTG TCA C 3'.

In the PCR reactions, the primer pairs consisted of either the two 16-mers derived from clone 81, or the 16-mer from clone 36 and the 16-mer from clone 37b.

The PCR reaction products were analyzed by separation of the products by alkaline gel electrophoresis, followed by Southern blotting, and detection of the amplified HCV-cDNA sequences with a 32 P-

35 labeled internal oligonucleotide probe derived from a

region of the HCV cDNA which does not overlap the primers. The PCR reaction mixtures were extracted with phenol/chloroform, and the nucleic acids precipitated from the aqueous phase with salt and ethanol. The precipitated nucleic acids were collected by centrifugation, and dissolved in distilled water. Aliquots of the samples were subjected to electrophoresis on 1.8% alkaline agarose gels. Single stranded DNA of 60, 108, and 161 nucleotide lengths were co-electrophoresed on the gels as molecular weight markers. After electrophoresis, the DNAs in the gel were transferred onto Biorad Zeta Probe* paper. Prehybridization and hybridization, and wash conditions were those specified by the manufacturer (Biorad).

The probes used for the hybridization-detection 15 of amplified HCV cDNA sequences were the following. the pair of PCR primers were derived from clone 81, the probe was an 108-mer with a sequence corresponding to that which is located in the region between the sequences of the two primers. When the pair of PCR primers were 20 derived from clones 36 and 37b, the probe was the nicktranslated HCV cDNA insert derived from clone 35. The primers are derived from nucleotides 155-170 of the clone 37b insert, and 206-268 of the clone 36 insert. The 3'end of the HCV cDNA insert in clone 35 overlaps 25 nucleotides 1-186 of the insert in clone 36; and the 5'end of clone 35 insert overlaps nucleotides 207-269 of the insert in clone 37b. (Compare Figs. 5, 8 and 10.) Thus, the cDNA insert in clone 35 spans part of the region between the sequences of the clone 36 and 37b derived primers, and is useful as a probe for the amplified sequences which include these primers.

Analysis of the RNA from the liver specimens was according to the above procedure utilizing both sets of primers and probes. The RNA from the liver of the three chimpanzees with NANBH yielded positive hybridization

results for amplification sequences of the expected size (161 and 586 nucleotides for 81 and 36 and 37b, respectively), while the control chimpanzees yielded negative hybridization results. The same results were achieved when the experiment was repeated three times.

Analysis of the nucleic acids and RNA from plasma was also according to the above procedure utilizing the primers and probe from clone 81. The plasmas were from two chimpanzees with NANBH, from a control chimpanzee, and pooled plasmas from control chimpanzees. Both of the NANBH plasmas contained nucleic acids/RNA which yielded positive results in the PCR amplified assay, while both of the control plasmas yielded negative results. These results have been repeatably obtained several times.

IV.D. Radioimmunoassay for Detecting HCV Antibodies in Serum from Infected Individuals

Solid phase radioimmunoassays to detect antibodies to HCV antigens were developed based upon Tsu and Herzenberg (1980). Microtiter plates (Immulon 2, Removawell strips) are coated with purified polypeptides containing HCV epitopes. The coated plates are incubated with either human serum samples suspected of containing 25 antibodies to the HCV epitopes, or to appropriate controls. During incubation, antibody, if present, is immunologically bound to the solid phase antigen. After removal of the unbound material and washing of the microtiter plates, complexes of human antibody-NANBV antigen are detected by incubation with 125 I-labeled sheep 30 anti-human immunoglobulin. Unbound labeled antibody is removed by aspiration, and the plates are washed. The radioactivity in individual wells is determined; the amount of bound human anti-HCV antibody is proportional to

35 the radioactivity in the well.

IV.D.1. Purification of Fusion Polypeptide SOD-NANB $_{5-1-1}$. The fusion polypeptide SOD-NANB $_{5-1-1}$, expressed in recombinant bacteria as described in Section IV.B.1., was purified from the recombinant $\underline{\mathbf{E}}$. $\underline{\mathrm{coli}}$ by differential extraction of the cell extracts with urea, followed by chromatography on anion and cation exchange columns as follows.

Thawed cells from 1 liter of culture were resuspended in 10 ml of 20% (w/v) sucrose containing 0.01M Tris HCl, pH 8.0, and 0.4 ml of 0.5M EDTA, pH 8.0 was 10 added. After 5 minutes at 0°C, the mixture was centrifuged at 4,000 x g for 10 minutes. The resulting pellet was suspended in 10 ml of 25% (w/v) sucrose containing 0.05 M Tris HCl, pH 8.0, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 microgram/ml pepstatin A, followed by addition of 0.5 ml lysozyme (10 mg/ml) and incubation at 0°C for 10 minutes. After the addition of 10 ml 1% (v/v) Triton X-100 in 0.05 M Tris HCl, pH 8.0, 1 mM EDTA, the mixture was incubated an ad-20 ditional 10 min at 0°C with occasional shaking. The resulting viscous solution was homogenized by passage 6 times through a sterile 20-gauge hypodermic needle, and centrifuged at 13,000 x g for 25 minutes. The pelleted material was suspended in 5 ml of 0.01 M Tris HCl pH 8.0, and the suspension centrifuged at 4,000 x g for 10 25 minutes. The pellet, which contained SOD-NANB $_{5-1-1}$ fusion protein, was dissolved in 5 ml of 6 M urea in 0.02 M Tris HCl, pH 8.0, 1 mM dithiothreitol (Buffer A), and was applied to a column of Q-Sepharose Fast Flow equilibrated with Buffer A. Polypeptides were eluted with a linear gradient of 0.0 to 0.3 M NaCl in Buffer A. After elution, fractions were analyzed by polyacrylamide gel electrophoresis in the presence of SDS to determine their content of SOD-NANB₅₋₁₋₁. Fractions containing this polypeptide were pooled, and dialyzed against 6 M urea in

0.02 M sodium phosphate buffer, pH 6.0, 1 mM dithiothreitol (Buffer B). The dialyzed sample was applied on a column of S-Sepharose Fast Flow equilibrated with Buffer B, and polypeptides eluted with a linear gradient of 0.0 to 0.3 M NaCl in Buffer B. The fractions were analyzed by polyacrylamide gel electrophoresis for the presence of SOD-NANB₅₋₁₋₁, and the appropriate fractions were pooled.

The final preparation of SOD-NANB₅₋₁₋₁

10 polypeptide was examined by electrophoresis on polyacrylamide gels in the presence of SDS. Based upon this analysis, the preparation was more than 80% pure.

IV.D.2. Purification of Fusion Polypeptide SOD-NANB 81.

The fusion polypeptide SOD-NANB₈₁, expressed in recombinant bacteria as described in Section IV.B.2., was purified from recombinant \underline{E} . $\underline{\operatorname{coli}}$ by differential extraction of the cell extracts with urea, followed by chromatography on anion and cation exchange columns utilizing the procedure described for the isolation of fusion polypeptide SOD-NANB₅₋₁₋₁ (See Section IV.D.1.).

The final preparation of SOD-NANB₈₁ polypeptide was examined by electrophoresis on polyacrylamide gels in the presence of SDS. Based upon this analysis, the preparation was more than 50% pure.

IV.D.3. <u>Detection of Antibodies to HCV Epitopes by Solid</u> Phase Radioimmunoassay.

Serum samples from 32 patients who were

30 diagnosed as having NANBH were analyzed by
radioimmunoassay (RIA) to determine whether antibodies to
HCV epitopes present in fusion polypeptides SOD-NANB₅₋₁₋₁
and SOD-NANB₈₁ were detected.

Microtiter plates were coated with SOD-NANE $_{5-1-1}$ or SOD-NANB $_{81}$, which had been partially purified according

to Sections IV.D.1. and IV.D.2., respectively. The assays were conducted as follows.

One hundred microliter aliquots containing 0.1 to 0.5 micrograms of SOD-NANB₅₋₁₋₁ or SOD-NANB₈₁ in 0.125 5 M Na borate buffer, pH 8.3, 0.075 M NaCl (BBS) was added to each well of a microtiter plate (Dynatech Immulon 2 Removawell Strips). The plate was incubated at 4°C overnight in a humid chamber, after which, the protein solution was removed and the wells washed 3 times with BBS 10 containing 0.02% Triton X-100 (BBST). To prevent nonspecific binding, the wells were coated with bovine serum albumin (BSA) by addition of 100 microliters of a 5 mg/ml solution of BSA in BBS followed by incubation at room temperature for 1 hour; after this incubation the BSA 15 solution was removed. The polypeptides in the coated wells were reacted with serum by adding 100 microliters of serum samples diluted 1:100 in 0.01M Na phosphate buffer, pH 7.2, 0.15 M NaCl (PBS) containing 10 mg/ml BSA, and incubating the serum containing wells for 1 hr at 37°C. 20 After incubation, the serum samples were removed by aspiration, and the wells were washed 5 times with BBST. $Anti-NANB_{5-1-1}$ and $Anti-NANB_{81}$ bound to the fusion polypeptides was determined by the binding of 125 I-labeled F'(ab), sheep anti-human IgG to the coated wells. 25 Aliquots of 100 microliters of the labeled probe (specific activity 5-20 microcuries/microgram) were added to each

Aliquots of 100 microliters of the labeled probe (specific activity 5-20 microcuries/microgram) were added to each well, and the plates were incubated at 37°C for 1 hour, followed by removal of excess probe by aspiration, and 5 washes with BBST. The amount of radioactivity bound in each well was determined by counting in a counter which detects gamma radiation.

The results of the detection of anti-NANB $_{\rm 5-1-1}$ and anti-NANB $_{\rm 81}$ in individuals with NANBH is presented in Table 1.

Table 1 Detection of Anti-5-1-1 and Anti-81 in Sera of NANB, HAV and HBV Hepatitis Patients

	Patient		S/N	
5	Reference Number	Diagnosis	Anti-5-1-1	
	ι. 201	Chronic NANB, IVD ² Chronic NANB, IVD Chronic NANB, IVD	0.77 1.14 2.11	4.20 5.14 4.05
10	2. 29 ¹	AVH ³ , NANB, Sporadic Chronic, NANB Chronic, NANB	1.09 33.89 36.22	1.05 11.39 13.67
	3. 30 ¹	AVH, NANB, IVD Chronic NANB, IVD Chronic N A NB, IVD	1.90 34.17 32.45	1.54 30.28 30.84
	4. 31	Chronic NANB, PT4	16.09	8.05
15	5. 32 ¹	Late AVH NANB, IVD Late AVH NANB, IVD	0.69 0.73	0.94 0.68
	6. 331	AVH, NANB, IVD AVH, NANB, IVD	1.66 1.53	1.96 0.56
20	7. 34 ¹	Chronic NAMB, PT Chronic NAMB, PT Chronic NAMB, PT Chronic NAMB, PT	34.40 45.55 41.58 44.20	7.55 13.11 13.45 15.48
	8. 35 ¹	AVH NANB, IVD "Healed" lecent NANB, AVH	31.92 6.87	31.95 4.45
	9. 36	Late AVH NAMB PT	11.84	5.79
25	10. 37	AVH NAMB, IVO	6.52	1.33
	11. 38	· Late AVH NAMB, PT	39.44	39.18
	12. 39	Chronic NANB, PT	. 42.22	37.54
	13. 40	AVH, NAMB, PT	1.35	1.17
30	14. 41	Chronic NAMB? PT	0.35	0.28

	Patient Reference		S/N	
	Number	Diagnosis	Anti-5-1-1	AIICI-01
	15. 42	AVH, NAMB, IVD	6.25	2.34
5	16. 43	Chronic NANB, PT	0.74	0.61
	17. 44	AVH, NANB, PT	5.40	1.83
	18. 45	Chronic, NANB, PT	0.52	0.32
	19. 46	AVH, NANB	23.35	4.45
10	20. 47	AVH, Type A	1.60	-1.35
	21. 48	AVH, Type A	1.30	0.66
	22. 49	AVH, Type A	1.44	0.74
	23. 50	Resolved Recent AVH, Type A	0.48	0.56
15	24 - 51	AVH, Type A Resolved AVH, Type A	0.68 0.80	0.64 0.65
	25. 52	Resolved Recent AVH, Type A Resolved Recent AVH, Type A	0.80	0.65
20	26. 53	AVH, Type A Resolved Recent AVH, Type A	1.85	1.16 0.88
	27. 54	AVH, Type A	1.35	0.74
	28. 55	Late AVH, HBV	0.58	0.55
25	29. 56	Chronic HBV	0.84	1.06
	30. 57	Late AVH, HBV	3.20	1.60
	31. 58	Chronic HBV	0.47	0.46
30	32. 59 ¹	AVH, HBV Healed AVH, HBV	0.73 0.43	0.60 0.44
30	33. 60 ¹	AVH, HBV Healed AVH, HBV	1.06 0.75	0.92 0.68

	Patient		S/	N
	Reference Number	Diagnosis	Anti-5-1-1	Anti-81
5	34. 611	AVH, HBV Healed AVH, HBV	. 1.66 0.63	0.61 0.36
	35. 62 ¹	AVH, HBV Healed AVH, HBV	1.02 0.41	0.73
10	16. 63 ¹	AVH, HBV Healed AVH, HBV	1.24 1.55	1.31
	17. 64 ¹	AVH, HBV Healed AVH, HBV	0.82 0.53	0.79 0.37
	38. 65 ¹	AVH, HBV Healed AVH, HBV	0.95 0.70	0.92 0.50
15	J9. 66 ¹	AVH, HBV Healed AVH, HBV	1.03	0.68 1.39

¹Sequential serum samples available from these patients ²tVD=Intravenus Drug User ³AVH=Acute viral hepatitis ⁴PT=Post transfusion

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As seen in Table 1, 19 of 32 sera from patients diagnosed as having NANBH were positive with respect to antibodies directed against HCV epitopes present in SOD-NANB $_{5-1-1}$ and SOD-NANB $_{81}$.

However, the serum samples which were positive were not equally immunologically reactive with SOD-NANB₅₋₁₋₁ and SOD-NANB₈₁. Serum samples from patient No. 1 were positive to SOD-NANB₈₁ but not to SOD-NANB₅₋₁₋₁. Serum samples from patients number 10, 15, and 17 were positive to SOD-NANB₅₋₁₋₁ but not to SOD-NANB₈₁. Serum samples from patients No. 3, 8, 11, and 12 reacted equally with both fusion polypeptides, whereas serum samples from patients No. 2, 4, 7, and 9 were 2-3 fold higher in the reaction to SOD-NANB₅₋₁₋₁ than to SOD-NANB₈₁. These results suggest that NANB₅₋₁₋₁ and NANB₈₁ may contain at least 3 different epitopes; i.e., it is possible that each polypeptide contains at least 1 unique epitope, and that the two polypeptides share at least 1 epitope.

IV.D.4. Specificity of the Solid Phase RIA for NANBH 20 The specificity of the solid phase RIAs for NANBH was tested by using the assay on serum from patients infected with HAV or with HBV and on sera from control individuals. The assays utilizing partially purified SOD- $NANB_{5-1-1}$ and $SOD-NANB_{81}$ were conducted essentially as described in Section IV.D.3, except that the sera was from patients previously diagnosed as having HAV or HBV, or from individuals who were blood bank donors. The results for sera from HAV and HBV infected patients are presented in table 1. The RIA was tested using 11 serum specimens from HAV infected patients, and 20 serum specimens from HBV infected patients. As shown in table 1, none of these sera yielded a positive immunological reaction with the fusion polypeptides containing BB-NANBV epitopes.

The RIA using the ${\rm NANB}_{5-1-1}$ antigen was used to determine immunological reactivity of serum from control individuals. Out of 230 serum samples obtained from the normal blood donor population, only 2 yielded positive reactions in the RIA (data not shown). It is possible that the two blood donors from whom these serum samples originated had previously been exposed to HCV.

10 IV.D.5. Reactivity of NANB₅₋₁₋₁ During the Course of NANBH Infection.

The presence of anti-NANB $_{5-1-1}$ antibodies during the course of NANBH infection of 2 patients and 4 chimpanzees was followed using RIA as described in Section IV.D.3. In addition the RIA was used to determine the presence or absence of anti-NANB $_{5-1-1}$ antibodies during the course of infection of HAV and HBV in infected chimpanzees.

show that with chimpanzees and with humans, anti-NANB₅₋₁₋₁ antibodies were detected following the onset of the acute phase of NANBH infection. Anti-NANB₅₋₁₋₁ antibodies were not detected in serum samples from chimpanzees infected with either HAV or HBV. Thus anti-NANB₅₋₁₋₁ antibodies serve as a marker for an individual's exposure to HCV.

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Table 2
Seroconversion in Sequential Serum Samples from Hepatitis Patients and Chimpanzees Using S-1-1 Antigen

•		•	
Sample Date (Days) (o=inoculation day)	Hepatitis Viruses	Anti-5-l-l (5/N)	ALT (mu/ml
T T	NANG	1 00	1180
T+180	112.5		425
1.200		36.22	
T	NANB	1.90	1830
T+307			290
T+799			216
		32.13	2.0
	NANB	0.87	ģ
		0.93	71
118		23.67	19
, 154			
		_	
	NANB	1.00	5
21		1.08	52
73		4.64	13
138		25.01	
	Navo	1 08	8
	ניאניט		
			205
			14
139		11.87	6
-3	BAAN	1.12	11
			132
		6 60	112
		17 51	
•••		61.31	
0	HAV	1.50	4
25			147
40			18
268			Š
_			-
	HAV	0.85	
			106
		0.81	10
129		1.33	
	Sample Date (Days) (0=inoculation day) T # 180 T+208 T # 180 T+208 T # 180	Sample Date (Days) Hepatitis (O=inoculation day) Viruses T	Sample Date (Days) Hepatitis Anti-5-1-1 (O=inoculation day) Viruses (S/N) T

5	Patient/ Chimp	Sample Date (Days) (o=inoculation day)	Hepatitis Viruses	Anti-5-1-1 (S/N)	ALT (mu/ml)
	Chimp 7	. 0 22 115 139	нач .	1.17 1.60 1.55 1.60	83 5
10	Chimp 8.	0 26 74 205	HAV	0.77 1.98 1.77 1.27	15 130 8 5
	Chimp 9	-290 379 435	HBV	1.74 3.29 2.77	9 6
15	Chimp 10	0 111-118 (pool) 205 240	KBV	2.35 2.74 2.05 1.78	96-156 (peril-
15	Chimp II	0 28-56 (pool) 169 223	нву	1.82 1.26 0.52	8-100 [pool:

20 *T=day of initial sampling

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IV.E. <u>Purification of Polyclonal Serum Antibodies to</u> NANB₅₋₁₋₁

On the basis of the specific immunological reactivity of the SOD-NANB $_{5-1-1}$ polypeptide with the antibodies in serum samples from patients with NANBH, a method was developed to purify serum antibodies which react immunologically with the epitope(s) in NANB $_{5-1-1}$. This method utilizes affinity chromatography. Purified SOD-NANB $_{5-1-1}$ polypeptide (see Section IV.D.1) was attached to an insoluble support; the attachment is such that the immobilized polypeptide retains its affinity for antibody to NANB $_{5-1-1}$. Antibody in serum samples is absorbed to the matrix-bound polypeptide. After washing to remove non-specifically bound materials and unbound materials, the bound antibody is released from the bound SOD-HCV polypeptide by change in pH, and/or by chaotropic reagents, for example, urea.

Nitrocellulose membranes containing bound SOD- $NANB_{5-1-1}$ were prepared as follows. A nitrocellulose 20 membrane, 2.1 cm Sartorius of 0.2 micron pore size, was washed for 3 minutes three times with BBS. $SOD-NANB_{5-1-1}$ was bound to the membrane by incubation of the purified preparation in BBS at room temperature for 2 hours; alternatively it was incubated at 4°C overnight. solution containing unbound antigen was removed, and the filter was washed three times with BBS for three minutes per wash. The remaining active sites on the membrane were blocked with BSA by incubation with a 5 mg/ml BSA solution for 30 minutes. Excess BSA was removed by washing the 30 membrane with 5 times with BBS and 3 times with distilled water. The membrane containing the viral antigen and BSA was then treated with 0.05 M glycine hydrochloride, pH 2.5, 0.10 M NaCl (GlyHCl) for 15 minutes, followed by 3 three minute washes with PBS.

Polyclonal anti-NANB₅₋₁₋₁ antibodies were isolated by incubating the membranes containing the fusion polypeptide with serum from an individual with NANBH for 2 hours. After the incubation, the filters were washed 5 times with BBS, and twice with distilled water. Bound antibodies were then eluted from each filter with 5 elutions of GlyHCl, at 3 minutes per elution. The pH of the eluates was adjusted to pH 8.0 by collecting each eluate in a test tube containing 2.0 M Tris HCl, pH 8.0.

Recovery of the anti-NANB₅₋₁₋₁ antibody after affinity chromatography is approximately 50%.

The nitrocellulose membranes containing the bound viral antigen can be used several times without appreciable decrease in binding capacity. To reuse the membranes, after the antibodies have been eluted the membranes are washed with BBS three times for 3 minutes. They are then stored in BBS at 4° C.

IV.F. The Capture of HCV Particles from Infected Plasma
Using Purified Human Polyclonal Anti-HCV Antibodies;
Hybridization of the Nucleic Acid in the Captured
Particles to HCV cDNA

IV.F.1. The Capture of HCV Particles from Infected Plasma Using Human Polyclonal Anti-HCV Antibodies

Protein-nucleic acid complexes present in infectious plasma of a chimpanzee with NANBH were isolated using purified human polyclonal anti-HCV antibodies which were bound to polystyrene beads.

Polyclonal anti-NANB $_{5-1-1}$ antibodies were purified from serum from a human with NANBH using the SOD-HCV polypeptide encoded in clone 5-1-1. The method for purification was that described in Section IV.E.

The purified anti-NANB $_{5-1-1}$ antibodies were bound to polystyrene beads (1/4" diameter, specular fin-

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ish, Precision Plastic Ball Co., Chicago, Illinois) by incubating each at room temperature overnight with 1 ml of antibodies (1 microgram/ml in borate buffered saline, pH 8.5). Following the overnight incubation, the beads were washed once with TBST [50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20], and then with phosphate buffered saline (PBS) containing 10 mg/ml BSA.

Control beads were prepared in an identical fashion, except that the purified anti-NANB $_{5-1-1}$ antibodies were replaced with total human immunoglobulin.

Capture of HCV from NANBH infected chimpanzee plasma using the anti-NANB $_{5-1-1}$ antibodies bound to beads was accomplished as follows. The plasma from a chimpanzee with NANBH used is described in Section IV.A.1.. An aliquot (1 ml) of the NANBV infected chimpanzee plasma was incubated for 3 hours at 37° C with each of 5 beads coated with either anti-NANB $_{5-1-1}$ antibodies, or with control immunoglobulins. The beads were washed 3 times with TBST.

20 IV.F.2. <u>Hybridization of the Nucleic Acid in the Captured</u> Particles to NANBV-cDNA

The nucleic acid component released from the particles captured with anti-NANB $_{5-1-1}$ antibodies was analyzed for hybridization to HCV cDNA derived from clone 81.

HCV particles were captured from NANBH infected chimpanzee plasma, as described in IV.F.1. To release the nucleic acids from the particles, the washed beads were incubated for 60 min. at 37°C with 0.2 ml per bead of a solution containing proteinase k (1 mg/ml), 10 mM Tris HCl, pH 7.5, 10 mM EDTA, 0.25% (w/v) SDS, 10 micrograms/ml soluble yeast RNA, and the supernatant solution was removed. The supernatant was extracted with phenol and chloroform, and the nucleic acids precipitated with ethanol overnight at -20°C. The nucleic acid precipitate

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was collected by centrifugation, dried, and dissolved in 50 mM Hepes, pH 7.5. Duplicate aliquots of the soluble nucleic acids from the samples obtained from beads coated with anti-NANB₅₋₁₋₁ antibodies and with control beads containing total human immunoglobulin were filtered onto to nitrocellulose filters. The filters were hybridized with a ³²P-labeled, nick-translated probe made from the purified HCV cDNA fragment in clone 81. The methods for preparing the probe and for the hybridization are described in Section IV.C.1..

Autoradiographs of a probed filter containing the nucleic acids from particles captured by beads containing anti-NANB₅₋₁₋₁ antibodies are shown in Fig. 40. The extract obtained using the anti-NANB₅₋₁₋₁ antibody (A_1,A_2) gave clear hybridization signals relative to the control antibody extract (A_3,A_4) and to control yeast RNA (B_1,B_2) . Standards consisting of lpg, 5pg, and l0pg of the purified, clone 81 cDNA fragment are shown in C1-3, respectively.

These results demonstrate that the particles captured from NANBH plasma by anti-NANB $_{5-1-1}$ -antibodies contain nucleic acids which hybridize with HCV cDNA in clone 81, and thus provide further evidence that the cDNAs in these clones are derived from the etiologic agent for NANBH.

IV.G. <u>Immunological Reactivity of C100-3 with Purified</u> Anti-NANB 5-1-1 Antibodies

The immunological reactivity of C100-3 fusion polypeptide with anti-NANB $_{5-1-1}$ antibodies was determined by a radioimmunoassay, in which the antigens which were bound to a solid phase were challenged with purified anti-NANB $_{5-1-1}$ antibodies, and the antigen-antibody complex detected with 125 I-labeled sheep anti-human antibodies.

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The immunological reactivity of C100-3 polypeptide was compared with that of SOD-NANB $_{5-1-1}$ antigen.

The fusion polypeptide C100-3 was synthesized and purified as described in Section IV.B.5. and in Section IV.B.6., respectively. The fusion polypeptide SOD-NANB₅₋₁₋₁ was synthesized and purified as described in Section IV.B.1. and in Section IV.D.1., respectively. Purified anti-NANB₅₋₁₋₁ antibodies were obtained as described in Section IV.E.

One hundred microliter aliquots containing 10 varying amounts of purified Cl00-3 antigen in 0.125M Na borate buffer, pH 8.3, 0.075M NaCl (BBS) was added to each well of a microtiter plate (Dynatech Immulon 2 Removawell Strips). The plate was incubated at 40C overnight in a 15 humid chamber, after which, the protein solution was removed and the wells washed 3 times with BBS containing 0.02% Triton X-100 (BBST). To prevent non-specific binding, the wells were coated with BSA by addition of 100 microliters of a 5 mg/ml solution of BSA in BBS followed 20 by incubation at room temperature for 1 hour, after which the excess BSA solution was removed. The polypeptides in the coated wells were reacted with purified anti-NANB $_{5-1-1}$ antibodies by adding 1 microgram antibody/well, and incubating the samples for 1 hr at 37°C. After incuba-25 tion, the excess solution was removed by aspiration, and the wells were washed 5 times with BBST. Anti-NANB $_{5-1-1}$ bound to the fusion polypeptides was determined by the binding of 125 I-labeled F'(ab), sheep anti-human IgG to the coated wells. Aliquots of 100 microliters of the labeled probe (specific activity 5-20 microcuries/ microgram) were added to each well, and the plates were incubated at 37°C for 1 hour, followed by removal of excess probe by aspiration, and 5 washes with BBST. The amount of radioactivity bound in each well was determined by counting in a counter which detects gamma radiation.

The results of the immunological reactivity of C100 with purified anti-NANB $_{5-1-1}$ as compared to that of NANB $_{5-1-1}$ with the purified antibodies are shown in Table 3.

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10	RIA (cpm/assay)						
	AG(ng)	400	320	240	160	60	0
	NANB 5-1-1	7332	6732	4954	4050	3051	57
15	C100-3	7450	6985	5920	5593	4096	67

The results in Table 3 show that anti-NANB $_{5-1-1}$ recognizes an epitope(s) in the C100 moiety of the C100-3 polypeptide. Thus NANB $_{5-1-1}$ and C100 share a common epitope(s). The results suggest that the cDNA sequence encoding this NANBV epitope(s) is one which is present in both clone 5-1-1 and in clone 81.

IV.H. Characterization of HCV

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IV.H.1. Characterization of the Strandedness of the HCV Genome.

The HCV genome was characterized with respect to its strandedness by isolating the nucleic acid fraction from particles captured on anti-NANB₅₋₁₋₁ antibody coated polystyrene beads, and determining whether the isolated nucleic acid hybridized with plus and/or minus strands of HCV cDNA.

Particles were captured from HCV infected States of the chimpanzee plasma using polystyrene beads coated with

immunopurified anti-NANB₅₋₁₋₁ antibody as described in Section IV.F.1. The nucleic acid component of the particles was released using the method described in Section IV.F.2. Aliquots of the isolated genomic nucleic 5 acid equivalent to 3 mls of high titer plasma were blotted onto nitrocellulose filters. As controls, aliquots of denatured HCV cDNA from clone 81 (2 picograms) was also blotted onto the same filters. The filters were probed with ³²P-labeled mixture of plus or mixture of minus 10 strands of single stranded DNA cloned from HCV cDNAs; the cDNAs were excised from clones 40b, 81, and 25c.

The single stranded probes were obtained by excising the HCV cDNAs from clones 81, 40b, and 25c with EcoRI, and cloning the cDNA fragments in M13 vectors, mp18 15 and mp19 [Messing (1983)]. The M13 clones were sequenced to determine whether they contained the plus or minus strands of DNA derived from the HCV cDNAs. Sequencing was by the dideoxychain termination method of Sanger et al. (1977).

Each of a set of duplicate filters containing aliquots of the HCV genome isolated from the captured particles was hybridized with either plus or minus strand probes derived from the HCV cDNAs. Fig. 41 shows the autoradiographs obtained from probing the NANBV genome 25 with the mixture of probes derived from clones 81, 40b, and 25c. This mixture was used to increase the sensitivity of the hybridization assay. The samples in panel I were hybridized with the plus strand probe mixture. The samples in panel II were probed by hybridization with the 30 minus strand probe mixture. The composition of the samples in the panels of the immunoblot are presented in table 4.

Table 4

		A	В
	lane		
5	. 1	HCV genome	*
	2		*
10	3	*	cDNA 81
	4		cDNA 81

* is an undescribed sample.

As seen from the results in Fig. 41, only the minus strand DNA probe hybridizes with the isolated HCV genome. This result, in combination with the result showing that the genome is sensitive to RNase and not DNase (See Section IV.C.2.), suggests that the genome of NANBV is positive stranded RNA.

These data, and data from other laboratories concerning the physicochemical properties of a putative NANBV(s), are consistent with the possibility that HCV is a member of the Flaviviridae. However, the possibility that HCV represents a new class of viral agent has not been eliminated.

30 IV.H.2. <u>Detection of Sequences in Captured Particles</u>
Which When Amplified by PCR Hybridize to HCV cDNA Derived from Clone 81

The RNA in captured particles was obtained as described in Section IV.H.1. The analysis for sequences which hybridize to the HCV cDNA derived from clone 81 was

carried out utilizing the PCR amplification procedure, as described in Section IV.C.3, except that the hybridization probe was a kinased oligonucleotide derived from the clone 81 cDNA sequence. The results showed that the amplified sequences hybridized with the clone 81 derived HCV cDNA probe.

IV.H.3. Homology Between the Non-Structural Protein of Dengue Flavivirus (MNWWVD1) and the HCV Polypeptides Encoded by the Combined ORF of Clones 14i Through 39c

The combined HCV cDNAs of clones 14i through 39c contain one continuous ORF, as shown in Fig. 26. The polypeptide encoded therein was analyzed for sequencehomology with the region of the non-structural 15 polypeptide(s) in Dengue flavivirus (MNWVD1). The analysis used the Dayhoff protein data base, and was performed on a computer. The results are shown in Fig. 42, where the symbol (:) indicates an exact homology, and the symbol (.) indicates a conservative replacement in the sequence; the dashes indicate spaces inserted into the sequence to achieve the greatest homologies. As seen from the figure, there is significant homology between the sequence encoded in the HCV cDNA, and the non-structural polypeptide(s) of Dengue virus. In addition to the homol-25 ogy shown in Fig. 42, analysis of the polypeptide segment encoded in a region towards the 3'-end of the cDNA also contained sequences which are homologous to sequences in the Dengue polymerase. Of consequence is the finding that the canonical Gly-Asp-Asp (GDD) sequence thought to be 30 essential for RNA-dependent RNA polymerases is contained in the polypeptide encoded in HCV cDNA, in a location which is consistent with that in Dengue 2 virus. not shown.)

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IV.H.4. HCV-DNA is Not Detectable in NANBH Infected Tissue

Two types of studies provide results suggesting
that HCV-DNA is not detectable in tissue from an
individual with NANBH. These results, in conjunction with
those described in IV.C. and IV.H.1. and IV.H.2. provide
evidence that HCV is not a DNA containing virus, and that
its replication does not involve cDNA.

IV.H.4.a. Southern Blotting Procedure

In order to determine whether NANBH infected chimpanzee liver contains detectable HCV-DNA (or HCV-cDNA), restriction enzyme fragments of DNA isolated from this source was Southern blotted, and the blots probed with \$^{32}P\$-labeled HCV cDNA. The results showed that the labeled HCV cDNA did not hybridize to the blotted DNA from the infected chimpanzee liver. It also did not hybridize to control blotted DNA from normal chimpanzee liver. In contrast, in a positive control, a labeled probe of the beta-interferon gene hybridized strongly to Southern blots of restriction enzyme digested human placental DNA. These systems were designed to detect a single copy of the gene which was to be detected with the labeled probe.

DNAs were isolated from the livers of two chimpanzees with NANBH. Control DNAs were isolated from uninfected chimpanzee liver, and from human placentas. The procedure for extracting DNA was essentially according to Maniatis et al. (1982), and the DNA samples were treated with RNAse during the isolation procedure.

Each DNA sample was treated with either EcoRI,

MboI, or HincII (12 micrograms), according to the
manufacturer's directions. The digested DNAs were
electrophoresed on 1% neutral agarose gels, Southern
blotted onto nitrocellulose, and the blotted material
hybridized with the appropriate nick-translated probe CDNA

(3 x 10 cpm/ml of hybridization mix). The DNA from

infected chimpanzee liver and normal liver were hybridized with ³²P-labeled HCV cDNA from clones 36 plus 81; the DNA from human placenta was hybridized with ³²P-labeled DNA from the beta-interferon gene. After hybridization, the blots were washed under stringent conditions, i.e., with a solution containing 0.1 x SSC, 0.1% SDS, at 65°C.

The beta-interferon gene DNA was prepared as described by Houghton et al (1981).

10 IV.H.4.b. Amplification by the PCR Technique

In order to determine whether HCV-DNA could be detected in liver from chimpanzees with NANBH, DNA was isolated from the tissue, and subjected to the PCR amplification-detection technique using primers and probe polynucleotides derived from HCV cDNA from clone 81. Negative controls were DNA samples isolated from uninfected HepG2 tissue culture cells, and from presumably uninfected human placenta. Positive controls were samples of the negative control DNAs to which a known relatively small amount (250 molecules) of the HCV cDNA insert from clone 81 was added.

In addition, to confirm that RNA fractions isolated from the same livers of chimpanzees with NANBH contained sequences complementary to the HCV-cDNA probe, the PCR amplification-detection system was also used on the isolated RNA samples.

In the studies, the DNAs were isolated by the procedure described in Section IV.H.4.a, and RNAs were extracted essentially as described by Chirgwin et al. 30 (1981).

Samples of DNA were isolated from 2 infected chimpanzee livers, from uninfected HepG2 cells, and from human placenta. One microgram of each DNA was digested with HindIII according to the manufacturer's directions. The digested samples were subjected to PCR amplification

and detection for amplified HCV cDNA essentially as described in Section IV.C.3., except that the reverse transcriptase step was omitted. The PCR primers and probe were from HCV cDNA clone 81, and are described in Section IV.C.3.. Prior to the amplification, for positive controls, a one microgram sample of each DNA was "spiked" by the addition of 250 molecules of HCV cDNA insert isolated from clone 81.

In order to determine whether HCV sequences were present in RNA isolated from the livers of chimpanzees with NANBH, samples containing 0.4 micrograms of total RNA were subjected to the amplification procedure essentially as described in Section IV.C.3., except that the reverse transcriptase was omitted from some of the samples as a negative control. The PCR primers and probe were from HCV cDNA clone 81, as described supra.

The results showed that amplified sequences complementary to the HCV cDNA probe were not detectable in the DNAs from infected chimpanzee liver, nor were they detectable in the negative controls. In contrast, when the samples, including the DNA from infected chimpanzee liver, was spiked with the HCV cDNA prior to amplification, the clone 81 sequences were detected in all positive control samples. In addition, in the RNA studies, amplified HCV cDNA clone 81 sequences were detected only when reverse transcriptase was used, suggesting strongly that the results were not due to a DNA contamination.

These results show that hepatocytes from

chimpanzees with NANBH contain no, or undetectable levels, of HCV DNA. Based upon the spiking study, if HCV DNA is present, it is at a level far below .06 copies per hepatocyte. In contrast, the HCV sequences in total RNA from the same liver samples was readily detected with the PCR technique.

IV.I. <u>ELISA Determinations for HCV Infection Using HCV</u> c100-3 As Test Antigen

All samples were assayed using the HCV c100-3 ELISA. This assay utilizes the HCV c100-3 antigen (which was synthesized and purified as described in Section IV.B.5), and a horseradish peroxidase (HRP) conjugate of mouse monoclonal anti-human IgG.

Plates coated with the HCV c100-3 antigen were prepared as follows. A solution containing Coating buffer (50mM Na Borate, pH 9.0), 21 ml/plate, BSA (25 micrograms/ 10 ml), c100-3 (2.50 micrograms/ml) was prepared just prior to addition to the Removeawell Immulon I plates (Dynatech Corp.). After mixing for 5 minutes, 0.2ml/well of the solution was added to the plates, they were covered and incubated for 2 hours at 37°C, after which the solution was removed by aspiration. The wells were washed once with 400 microliters Wash Buffer (100 mM sodium phosphate, pH 7.4, 140 mM sodium chloride, 0.1% (W/V) casein, 1% (W/ V) Triton x-100, 0.01% (W/V) Thimerosal). After removal 20 of the wash solution, 200 microliters/well of Postcoat solution (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, 0.1% (w/v) casein and 2 mM phenylmethylsulfonylfluoride (PMSF)) was added, the plates were loosely covered to prevent evaporation, and were al-25 lowed to stand at room temperature for 30 minutes. The wells were then aspirated to remove the solution, and lyophilized dry overnight, without shelf heating. The prepared plates may be stored at 2-8°C in sealed aluminum pouches.

In order to perform the ELISA determination, 20 microliters of serum sample or control sample was added to a well containing 200 microliters of sample diluent (100 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM EDTA, 0.1% (W/V) Casein, 0.015 (W/V) Therosal, 1% (W/V) Triton X-100, 100 micrograms/ml yeast extract). The

plates were sealed, and incubated at 37°C for two hours, after which the solution was removed by aspiration, and the wells were washed with 400 microliters of wash buffer (phosphate buffered saline (PBS) containing 0.05% Tween The washed wells were treated with 200 microliters of mouse anti-human IgG-HRP conjugate contained in a solution of Ortho conjugate diluent (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, 50% (V/V) fetal bovine serum, 1% (V/V) heat treated horse serum, 1 mM $K_{3}Fe(CN)_{6}$, 10 0.05% (W/V) Tween 20, 0.02% (W/V) Thimerosal). Treatment was for 1 hour at 37°C, the solution was removed by aspiration, and the wells were washed with wash buffer, which was also removed by aspiration. To determine the amount of bound enzyme conjugate, 200 microliters of 15 substrate solution (10 mg O-phenylenediamine dihydrochloride per 5 ml of Developer solution) was added. Developer solution contains 50 mM sodium citrate adjusted to pH 5.1 with phosphoric acid, and 0.6 microliters/ml of 30% H₂O₂. The plates containing the substrate solution were incubated in the dark for 30 minutes at room temperature, the reactions were stopped by the addition of 50 microliters/ml 4N sulfuric acid, and the ODs determined.

The examples provided below show that the

25 microtiter plate screening ELISA which utilizes HCV c100-3
antigen has a high degree of specificity, as evidenced by
an initial rate of reactivity of about 1%, with a repeat
reactive rate of about 0.5% on random donors. The assay
is capable of detecting an immunoresponse in both the post
30 acute phase of the infection, and during the chronic phase
of the disease. In addition, the assay is capable of
detecting some samples which score negative in the surrogate tests for NANBH; these samples come from
individuals with a history of NANBH, or from donors

implicated in NANBH transmission.

In the examples described below, the following abbreviations are used:

ALT Alanine amino transferase 5 Anti-HBc Antibody against HBC Antibody against HBsAg Anti-HBsAg Hepatitis B core antigen HBC Hepatitis B surface antigen ABsAq Immunoglobulin G IgG Immunoglobulin M 10 IgM International units/Liter IU/L Not available NA Not tested NT Sample size N Negative Neg 15 Optical density OD Positive Pos S/CO Signal/cutoff SD Standard deviation Average or mean 20 х Within normal limits WNL

IV.I.1. <u>HCV Infection in a Population of Random Blood</u> Donors

A group of 1,056 samples (fresh sera) from random blood donors were obtained from Irwin Memorial Blood Bank, San Francisco, California. The test results obtained with these samples are summarized in a histogram showing the distribution of the OD values (Fig. 43). As seen in Fig. 43, 4 samples read >3, 1 sample reads between 1 and 3, 5 samples read between 0.4 and 1, and the remaining 1,046 samples read <0.4, with over 90% of these samples reading <0.1.

The results on the reactive random samples are presented in Table 5. Using a cut-off value equal to the

mean plus 5 standard deviations, ten samples out of the 1,056 (0.95%) were initially reactive. Of these, five samples (0.47%) repeated as reactive when they were assayed a second time using the ELISA. Table 5 also shows the ALT and Anti-HBd status for each of the repeatedly reactive samples. Of particular interest is the fact that all five repeat reactive samples were negative in both surrogate tests for NANBH, while scoring positive in the HCV ELISA.

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TABLE 5 RESULTS ON REACTIVE RANDOM SAMPLES

N = 1051 $\bar{x} = 0.049 *$ $SD = \pm 0.074$ Cut-off: \bar{x} + 5SD = 0.419 (0.400 + Negative Control)

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		Initial Reactives	Repeat Reactives		Anti
	Samples	OD	OD	ALT**	<u>HBc***</u> (OD)
	4227	0.462	0.084	NA	NA
	6292	0.569	0.294	NA	NA
15	6188	0.699	0.326	NA	NA
	6157	0.735	0.187	NA	NA
	6277	0.883	0.152	NA	NA
	6397	1.567	1.392	30.14	1.433
	6019	>3.000	>3.000	46.48	1.057
	6651	>3.000	>3.000	48.53	1.343
	6669	>3.000	>3.000	60.53	1.165
20	4003	>3.000	3.000	WNL***	Negative
	10	/1056 = 0.95%	5/1056 = 0.47%		_

^{*} Samples reading >1.5 were not included in calculating the Mean and SD

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^{**} ALT ≥ 68 IU/L is above normal limits.

*** Anti-HBc ≤ 0.535 (competition assay) is considered positive. 25 **** WNL: Within normal limits.

IV.I.2. Chimpanzee Serum Samples

Serum samples from eleven chimpanzees were tested with the HCV c100-3 ELISA. Four of these chimpanzees were infected with NANBH from a contaminated batch of Factor VIII (presumably Hutchinson strain), following an established procedure in a collaboration with Dr. Daniel Bradley at the Centers for Disease Control. As controls, four other chimpanzees were infected with HAV and three with HBV. Serum samples were obtained at different times after infection.

The results, which are summarized in Table 6, show documented antibody seroconversion in all chimpanzees infected with the Hutchinson strain of NANBH. Following 15 the acute phase of infection (as evidenced by the significant rise and subsequent return to normal of ALT levels), antibodies to HCV c100-3 became detectable in the sera of the 4/4 NANBH infected chimpanzees. These samples had previously been shown, as discussed in Section 20 IV.B.3., to be positive by a Western analysis, and an RIA. In contrast, none of the control chimpanzees which had been infected with HAV or HBV showed evidence of reactivity in the ELISA.

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TABLE 6
CHIMPANZEE SERUM SAMPLES

		00	<u>\$/co</u>	IHOCULATIO DATE	DATE	ALT (IU/L)	TRANSFUSED
5	NEGATIVE CONTROL POSITIVE CONTROL CUTOFF	0.001 1.504 0.401			•		
10	Chimp I	-0.007 0.003 >3.000 >3.000	0.00 0.01 >7.48 >7.48	05/24/84	05/24/84 08/07/81 09/18/84 10/24/84	9 71 19	Nanb
	Chimp 2	 -0.003 -0.005 0.945 >3.000	0.00 0.00 2.36 >7.48	06/07/84	05/31/84 06/28/84 08/20/84 10/24/84	- 5 52 13	BIAN
15	Chimp 3	0.005 0.017 0.006 1.010	0.01 0.04 0.01 2.52	03/14/85	03/14/85 04/26/85 05/06/85 08/20/85	8 205 14 6	илив
20	Chimp 4	-0.006 0.003 0.523 1.574	0.00 0.01 1.31 3.93	03/11/85	03/11/85 05/09/85 06/06/85 08/01/85	11 132 	NANB
25	Chimp 5	-0.006 0.001 0.003 0.006	00.0 00.0 10.0	11/21/80	11/21/80 12/16/80 12/30/80 07/29 - 08/21/81	4 147 18 5	нлч
	Chimp 6	 -0.005 0.001 -0.004 0.290	0.00 0.00 0.00 0.72	05/25/82	05/17/82 06/10/82 07/06/82 10/01/82	106	нлv
30	Chimp 7	-0.008 -0.004 -0.005 0.005	0.00 0.00 0.00 0.01	05/ <i>2</i> 5/82	05/25/82 05/17/82 09/16/82 10/09/82	7 83 · 5	Нлч

TABLE 6
CHIMPANZEE SERUM SAMPLES

(Cont'd)

5		_00_	<u>s/co</u>	INOCULATION DATE	BLEED DATE	ALT (IU/L)	TRANSFUSED
	Chimp ·8	-0.007	0.00	11/21/30	11/21/80	15	IWA
		0.000	0.00		12/16/80	130	
		0.004	0.01		02/03/81	8	
		0.000	0.00		06/03 - 06/10/81	4.5	
10							
	Chimp 9			07/24/80			IIBV
		0.019	0.05		08/22 - 10/10/79		
					03/11/81	57	
		0.015	0.01		07/01 - 08/05/81	9	
		0.008	0.02		10/01/81	6	
							•
15	Chimp 10			05/12/82			HBV
		0.011	0.03		04/21 - 05/12/82	9	
		0.015	0.04		09/01 - 09/08/82	126	
		0.008	0.02		12/02/82	9	
		0.010	0.02		01/06/83	13	

	Chimp 11			05/12/82			IIBY
20	<u> </u>	0.000	0.00		01/06 - 05/12/82	11	
					06/23/82	100	
		-0.003	0.00		06/09 - 07/07/82		
		-0.003	0.00		10/28/82	9	
		-0.003	0.00		12/20/82	10	

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IV.I.3. Panel 1: Proven Infectious Sera from Chronic Human NANBH Carriers

A coded panel consisted of 22 unique samples, each one in duplicate, for a total of 44 samples. The 5 samples were from proven infectious sera from chronic NANBH carriers, infectious sera from implicated donors, and infectious sera from acute phase NANBH patients. addition, the samples were from highly pedigreed negative controls, and other disease controls. This panel was provided by Dr. H. Alter of the Department of Health and Human Services, National Institutes of Health, Bethesda, Maryland. The panel was constructed by Dr. Alter several years ago, and has been used by Dr. Alter as a qualifying panel for putative NANBH assays.

The entire panel was assayed twice with the ELISA assay, and the results were sent to Dr. Alter to be scored. The results of the scoring are shown in Table 7. Although the Table reports the results of only one set of . duplicates, the same values were obtained for each of the 20 duplicate samples.

As shown in Table 7, 6 sera which were proven infectious in a chimpanzee model were strongly positive. The seventh infectious serum corresponded to a sample for an acute NANBH case, and was not reactive in this ELISA. A sample from an implicated donor with both normal ALT levels and equivocal results in the chimpanzee studies was non-reactive in the assay. Three other serial samples from one individual with acute NANBH were also nonreactive. All samples coming from the highly pedigreed 30 negative controls, obtained from donors who had at least 10 blood donations without hepatitis implication, were non-reactive in the ELISA. Finally, four of the samples tested had previously scored as positive in putative NANBH assays developed by others, but these assays were not

confirmable. These four samples scored negatively with the HCV ELISA.

TABLE 7
H. ALTER'S PANEL 1:

5		Barrer	1 0	2 0
	1) P	PANEL ROYEN INFECTIOUS BY CHIMPANZEE TRANSMISSION	1ST RESULT	2ND RESULT
		. Chronic NANB: Post-TX		
	•	JF	•	•
		EB	•	.
		rg	+	+
	· 8	. IMPLICATED DOMORS WITH ELEVATED ALT		
10		BC	+	+
		11	+	+
		88	+	+
	C.	. Acute NANB: Post-Tx		
		Wil	-	- '
		DUIYOCALLY ENFECTIOUS BY CHIHPANZEE TRANSHISSION		
	Α.	. IMPLICATED DONOR WITH NORMAL ALT		
. 15			-	-
		CUTE NAMB: POST-TX		
		L VEEK L	-	-
		_ WEEK 2 _ NEEK 3	-	-
			-	•
		SEASE CONTROLS		•
20	٨.	PRIHARY BILIARY CIRRHOSIS		
20	•	EK	-	-
	. 8.	ALCOHOLIC HEPATITIS IN RECOVERY		
	C) 0-	IIB	-	-
	5) PE DH	DIGREED NEGATIVE CONTROLS	_	_
	DC		_	<u>-</u>
	LV	•	_	-
25	HL		_	-
23	ΛII		_	-
		TENTIAL NAVB "ANTIGENS"		
		G-80-017-0 (ISHIDA)	-	. •
		TERIX (TREPO)	-	-
		RTZ (ARNOLD)	-	-
		CASSDINE (TREPO)	-	-
30				

IV.I.4. Panel 2: Donor/Recipient NANBH

The coded panel consisted of 10 unequivocal donor-recipient cases of transfusion associated NANBH, with a total of 188 samples. Each case consisted of samples of some or all the donors to the recipient, and of serial samples (drawn 3, 6, and 12 months after transfusion) from the recipient. Also included was a prebleed, drawn from the recipient before transfusion. The coded panel was provided by Dr. H. Alter, from the NIH, and the results were sent to him for scoring.

The results, which are summarized in Table 8, show that the ELISA detected antibody seroconversion in 9 of 10 cases of transfusion associated NANBH. Samples from 15 case 4 (where no seroconversion was detected), consistently reacted poorly in the ELISA. Two of the 10 recipient samples were reactive at 3 months post transfusion. At six months, 8 recipient samples were reactive; and at twelve months, with the exception of case 4, all 20 samples were reactive. In addition, at least one antibody positive donor was found in 7 out of the 10 cases, with case 10 having two positive donors. Also, in case 10, the recipient's pre-bleed was positive for HCV antibodies. The one month bleed from this recipient dropped to border-25 line reactive levels, while it was elevated to positive at 4 and 10 month bleeds. Generally, a S/CO of 0.4 is considered positive. Thus, this case may represent a prior infection of the individual with HCV.

The ALT and HBc status for all the reactive,

i.e., positive, samples are summarized in Table 9. As
seen in the table, 1/8 donor samples was negative for the
surrogate markers and reactive in the HCV antibody ELISA.
On the other hand, the recipient samples (followed up to
12 months after transfusion) had either elevated ALT,

positive Anti-HBc, or both.

TABLE 8 DONOR/RECIPIENT NAMB PANEL

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H. ALTER DONOR/RECIPIENT NAMB PANEL

10	Case		10R <u>\$/C0</u>	RECTI PREB	PTENT LEED S/CO	3 Han	THSS/CO	Post- 6 How 00	*	12 Kon 00	THS S/C0
15	1.			.032	0.07 0.14	.112 .050	0.26 0.12	>3.000 1.681	>6.96 3.90	>3.000 >3.000	>6.96 >6.96
13	3.	.403	0.94	.049	0.11 0.15	.057	0.13	>3.000 .067	>6.96 0.16	>3.000 .217	>6.96 0.50
	4. 5.	>3.000 >3.000	>6.96 >6.96	.034	0.08	.096 1.475	0.22 3.44	>3.000 >3.000	>6.96 >6.96	>3.000 >3.000	>6.96 >6.95
20		>3.000 >3.000	>6.96 >6.96	.034	0.08	.056	0.13	>3.000 2.262	>6.96 5.28	>3.000 >3.000	>6.96 >6.96
	8. 9.	>3.000 >3.000 >3.000	>6.96 >6.96	.080	0.19	.127 .317*	0.30	.055 >3.000**	0.13 >6.96	>3.000 >3.000***	>6.96 >6.96
	10.	>3.000	>6.96	3,000	••••						

* 1 MONTH. ** 4 MONTHS. *** 10 MONTHS

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TABLE 9

ALT AND HBC STATUS FOR REACTIVE SAMPLES IN

H. ALTER PANEL 1

			Anti-	
	Sample	s	ALT*	HBc**
5	<u> </u>			
	Donors			
	Case 3		Normal	Negative
	Case 5		Elevated	Positive
	Case 6		Elevated	Positive
	Case 7		Not available	Negative
10	Case 8		Normal	Positive
	Case 9		Elevated	Not available
	Case 10		Normal	Positive
	Case 10		Normal	Positive
	Recipien	<u>ts</u>		
15	Case 1	6 то	Elevated	Positive
13	. 12 mo	Elevated	Not tested	
	Case 2	6 mo	Elevated	Negative
	12 mo	Elevated	Not tested	
	Case 3	6 mo	Normal	Not tested***
20	12 mo	Elevated	Not tested***	
	Case 5	6 mo	Elevated	Not tested
	· 12 mo	Elevated	Not tested	
	Case 6	3 mo	Elevated	Negative
	6 mo	Elevated	Negative	
25	12 mo	Elevated	Not tested	
	Case 7	6 mo	Elevated	Negative
	12 mo	Elevated	Negative	
	Case 8	6 mo	Normal	Positive
	12 mo	Elevated	Not tested	
30	Case 9	12 mo	Elevated	Not tested
	Case 10	4 mo	Elevated	Not tested
	10 mo	Elevated	Not tested	

ALT ≥45 IU/L is above normal limits.
 Anti-HBc ≤50% (competition assay) is considered positive.
 Prebleed and 3 mo samples were negative for HBc. 35

IV.I.5. <u>Determination of HCV Infection in High Risk Group</u> Samples

Samples from high risk groups were monitored using the ELISA to determine reactivity to HCV c100-3 antigen. These samples were obtained from Dr. Gary Tegtmeier, Community Blood Bank, Kansas City. The results are summarized in Table 10.

As shown in the table, the samples with the

10 highest reactivity are obtained from hemophiliacs (76%).

In addition, samples from individuals with elevated ALT
and positive for Anti-HBC, scored 51% reactive, a value
which is consistent with the value expected from clinical
data and NANBH prevalence in this group. The incidence of

15 antibody to HCV was also higher in blood donors with
elevated ALT alone, blood donors positive for antibodies
to Hepatitis B core alone, and in blood donors rejected
for reasons other than high ALT or anti-core antibody when
compared to random volunteer donors.

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TABLE 10

NANBH HIGH RISK GROUP SAMPLES

٦.	
_	

Group	N	Dist N	cribution OD	% Reactive
Elevated ALT	35 0.728	3	>3.000	11.4%
Anti-HBc	24	5	>3.000	20.8%
Elevated ALT, Anti-HBc	33 2.768 2.324 0.939 0.951 0.906	12	>3.000	51.5%
Rejected Donors	25	5	>3.000	20.0%
Donors with History of Hepatiti 1 1 1	150 0.837 0.714 0.469	19	>3.000	14.7%
Haemophiliacs 1 1 1 1 1 1 1	50 2.568 2.483 2.000 1.979 1.495 1.209 0.819	31	>3.000	76.0%

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IV.I.6 Comparative Studies Using Anti-IgG or Anti-IgM Monoclonal Antibodies, or Polyclonal Antibodies as a Second Antibody in the HCV c100-3 ELISA

The sensitivity of the ELISA determination which uses the anti-IgG monoclonal conjugate was compared to that obtained by using either an anti-IgM monoclonal conjugate, or by replacing both with a polyclonal antiserum reported to be both heavy and light chain 10 specific. The following studies were performed.

IV.I.6.a. Serial Samples from Seroconverters

Serial samples from three cases of NANB seroconverters were studied in the HCV c100-3 ELISA assay 15 using in the enzyme conjugate either the anti-IgG monoclonal alone, or in combination with an anti-IgM monoclonal, or using a polyclonal antiserum. The samples were provided by Dr. Cladd Stevens, N.Y. Blood Center, N.Y.C., N.Y.. The sample histories are shown in Table 11.

The results obtained using an anti-IgG monoclonal antibody-enzyme conjugate are shown in Table 12. The data shows that strong reactivity is initially detected in samples 1-4, 2-8, and 3-5, of cases 1, 2, and 3, respectively.

The results obtained using a combination of an 25 anti-IgG monoclonal conjugate and an anti-IgM conjugate are shown in Table 13. Three different ratios of anti-IgG to anti-IgM were tested; the 1:10,000 dilution of anti-IgG was constant throughout. Dilutions tested for the anti-30 IgM monoclonal conjugate were 1:30,000, 1:60,000, and 1:120,000. The data shows that, in agreement with the studies with anti-IgG alone, initial strong reactivity is detected in samples 1-4, 2-8, and 3-5.

The results obtained with the ELISA using anti-IgG monoclonal conjugate (1:10,000 dilution), or Tago

polyclonal conjugate (1:80,000 dilution), or Jackson polyclonal conjugate (1:80,000 dilution) are shown in Table 14. The data indicates that initial strong reactivity is detected in samples 1-4, 2-8, and 3-5 using all three configurations; the Tago polyclonal antibodies yielded the lowest signals.

The results presented above show that all three configurations detect reactive samples at the same time after the acute phase of the disease (as evidenced by the ALT elevation). Moreover, the results indicate that the sensitivity of the HCV c100-3 ELISA using anti-IgG monoclonal-enzyme conjugate is equal to or better than that obtained using the other tested configurations for the enzyme conjugate.

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TABLE 11

DESCRIPTION OF SAMPLES FROM CLADD STEVENS PANEL

5	 	Date	HBSAg	Anci-HBs	Anti-HBc	ALT	Biliruoin
	Case 1						
	1-1	8/5/81	1.0	91.7	12.9	40.0	-1.0
10	1-2	9/2/81	1.0	121.0	15.1	274.0	
10	1-3	10/7/81	1.0	64.0	23.8	261.0	
	1-4	. 11/19/81	1.0	67.3	33.8	75.0	
	.1-5	12/15/81	1.0	50.5	27.6	71.0	
	Case 2						•
15	•						
	2-1	10/19/81	1.0	1.0	116.2	17.0	-1.0
	2-2	11/17/81	1.0	0.8	89.5	46.0	1.1
	2-3	12/02/81	1.0	1.2	75.3	63.0	
-	2-4	12/14/81	1.0	0.9	90.6	132.0	
	2-5	12/23/81	1.0	0.8	93.6	624.0	
20	2-6	1/20/82	1.0	0.8	92.9	66.0	
	2-7	2/15/82	1.0	0.8	86.7	70.0	
	2-8	3/17/82	1.0	0.9	69.8	24.0	
	2-9	4/21/82	1.0	0.9	67.1	53.0	
	2-10	5/19/82	1.0	0.5	74.8	95.0	
	2-11	6/14/82	1.0	0.8	82.9	37.0	
25							
	Case 3						
	3-1	4/7/81	1.0	1.2	. 88.4	13.0	-1.0
	3-2	5/12/81	1.0	1.1	126.2	236.0	0.4
	3-3	5/30/81	1.0	0.7	99.9	471.0	0.2
30	3-4	6/9/81	1.0	1.2	110.8	315.0	
	3-5	7/6/81	1.0	1.1	89.9	273.0	
	3-6	8/10/81	1.0	1.0	118.2	158.0	
	3-7	9/8/81	1.0	1.0	112.3	84.0	
	. 3-8	10/14/81	1.0	0.9	102.5	180.0	(
-35	3-9	11/11/81	1.0	1.0	84.6	154.0	

TABLE 12

ELISA RESULTS OBTAINED USING AN ANTI-IGG MONOCLONAL CONJUGATE

5		SAMPLE	DATE	. <u>ALT</u>	<u>00</u>	 <u>S/C0</u>
		NEG CONTROL CUTOFF PC (1:128)			.076 .476 1.390	
10		CASE #1				
		1-1	08/05/81	40.0	.178	.37
		1-2	09/02/81	274.0	.154	.32
		i-3	10/07/81	261.0	.129	.27
		1-4	11/19/81	75.0	.937	1.97
	•	1-5	12/15/81	71.0	>3.000	>6.30
15	·	CASE #2				
		2-1	10/19/81	17.0	.058	0.12
	•	2-2	11/17/81 -	46.0	.050	0.11
		2-3	12/02/81	63.0	.047	0.10
		2-4	12/14/81	152.0	.059	0.12
		z-5	12/23/81	624.0	.070	0.15
20		2-6	01/20/82	66.0	.051	0.11
		2-7	02/15/82	70.0	.139	0.29
		2-8	03/17/82	24.0	1.867	3.92
		2-9	04/21/82	53.0	>3.000	>6.30
		2-10	05/19/82	95.0	>3.000	>6.30
		2-11	06/14/82	37.0	>3.000	>6.30
25		CASE #3				
		3-1	04/07/81	13.0	.090	.19
	-	3-2	05/12/81	236.0	.064	.13
		3-3	05/30/81	471.0	.079	.17
		3-4	06/09/81	315.0	.211	.44
		3-5	07/06/81	273.0	1.707	3.59
30		3-6	08/10/81	158.0	>3.000	>6.30
		3-7	09/08/81	84.0	>3.000	>6.30
		3-8	10/14/81	180.0	>3.000	>6.30
		3-9	11/11/81	154.0	>3.000	>6.30

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TABLE 13

ELISA RESULTS OBTAINED USING ANTI-IGG and ANTI-IGM

MONOCLONAL CONJUGATE

5	•				NAMB ELISAS		
				HONOCLONALS IGG 1:10K IGH 1:30K	IGG 1:10K		
	SAMPLE	DATE	<u>ALT</u>	<u>oo s/co</u>	<u>00 S/CO</u>	<u>00 S/C0</u>	
10	NEG CONTRO CUTOFF			-100	.080	.079	
	PC (1:128))		1.083	1.328	1.197	
	CASE #1			·			
	1-1	08/05/81	40	.173	.152	.070	
7.5	1-2	09/02/81	274	.194	.141	.079	
15	1-3	10/07/81	261	.162	.129	.063	
	1-4	11/19/81	75	. 312	.85	.709	
	1-5	12/15/81	71	>3.00	>3.00	>3.00	
	Case #2						
	2-1	10/19/81	17	.442	045	005	
20	2-2	11/17/81			.045	.085	
			46	.102 .	.029	.030	
	2-3	12/02/81	63	.059	.035	.027	
	2-4	12/14/81	152	.065	.041	.025	
•	2-5	12/23/81	624	.082	.033	.032	
	2-6	01/20/82	66	.102	.042	-027	
	2-7	02/15/82	70	.188	.068	.096	
25	2-8	03/17/82	24	1.728	1.668	1.541	
25	2-9	09/21/82	53	>3.00	2.443	>3.00	
	2-10	05/19/82	95	>3.00	>3.00	>3.00	
	2-11	06/14/82	37	>3.00	>3.00	>3.00	
	CASE #3						
30	3-1	04/07/81	13	.193	075		
	3-2	05/12/81	235	.201	.076	.049	
	3-3	05/30/81	471		:051	.038	
	3-4	06/09/81	315	.132	.067	.052	
	3-5	07/05/81		.175	.155	.140	,
•	3-6		273	1.335	1.238	1.260	
	3-7	18/01/80	158	>3.00	>3.00	>3.00	
		09/08/81	84	>3.00	>3.00	>3:00	á
35	3-8	10/14/81	180	>3.00	>3.00	>3.00	•
	3-9	11/11/81	154	>3.00	>3.00	>3.00	

TABLE 14

ELISA RESULTS OBTAINED USING POLYCIONAL CONJUGATES

_						NANB EL	.ISAs		
5				_	HONOCL		TAGO	JA	CKSON
					1:10		1:80K	i	:80K
٠	SAHPLE	DATE	ALT	_00	<u> 5/</u>	<u>'CO OC</u>	<u>s/co</u>	00	<u>s/co</u>
	NEG CON	TROL		.076	,	.045	i	.154	
	Cutoff			. 476		.545	;	.654	
10	PC (1:12	28)		1.390	!	<u>.727</u>		2.154	
	CASE #1								
	1-1	08/05/81	40	.178				.153	.23
	1-2	09/02/81	274	.154				.225	. 34
	1-3	10/07/81	261	.129	•27			.167	.26
	1-4	11/19/81	75	.937	1.97	.324	.60	.793	1.21
15	1-5	12/15/81	71	>3.00	>6.30	1.778	3.27	>3.00	>4.59
	CASE #2								
	2-i	10/19/81	17	.058				.052	.08
	2-2	11/17/81	46	.050		810.		.058	.09
	2-3	12/02/81	63	.047	.10	.020		.060	.09
20	2-4	12/14/81	152	.059	.12	.025		.054	.08
	2-5	12/23/81	624	.070	.15	.026	.05	.074	.11
	2-6	01/20/82	. 66	.051	.11	.018	.03	.058	.09
	2-7	02/15/82	70	.139	.29	.037	.07	.146	.22
	2-8 2-9	03/17/82	24	1.867	3.92	.355	.65	1.429	2.19
		04/21/82	53	>3.00	>6.30	.748	1.37	>3.00	>4.59
25	2-10 2-11	05/19/82 05/14/82	95 37	>3.00 >3.00	>6.30 >6.30	1.025	1.88	>3.00	>4.59
25		00/14/02	3/	23.00	>0.30	.917	. 1.68	>3.00	>4.59
	CASE #3								•
	3-1	04/07/81	13	.090	.19	.049	09	.138	.21
	3-2	05/12/81	235	.064	.13	.040	.07	.094	.19
	3-3	05/30/81	471	.079	.17	.045	.08	.144	.22
30	3-4 7-5	06/09/81	315	.211	.44	.085	.16	.275	.42
	3-5 3-6	07/06/81	273	1.707	3.59	.272	.50	1.773	2.71
	3-0 3-7	08/10/81	158	>3.00 >3.00	>6.30	1.347	2.47 4.21	>3.00	>4.59 >4.59
	3-7 3-8	18/80/20 10/14/81	84	>3.00 >3.00	>6.30 >6.30	2.294 >3.00	4.21 >5.50	>3.00	>4.59 >4.59
	3-8 3-9	11/11/81	180 154	>3.00 >3.00	>6.30			>3.00	>4.59 >4.59
	7-3	11/11/01	124	~J.UU	~0. 30	>3.00	>5.50	>3.00	, ~4.33

IV.I.6.b. Samples from Random Blood Donors

Samples from random blood donors (See Section IV.I.1.) were screened for HCV infection using the HCV c100-3 ELISA, in which the antibody-enzyme conjugate was either an anti-IgG monoclonal conjugate, or a polyclonal conjugate. The total number of samples screened were 1077 and 1056, for the polyclonal conjugate and the monoclonal conjugate, respectively. A summary of the results of the screening is shown in Table 15, and the sample distributions are shown in the histogram in Fig. 44.

The calculation of the average and standard deviation was performed excluding samples that gave a signal over 1.5, i.e., 1073 OD values were used for the calculations utilizing the polyclonal conjugate, and 1051 for the anti-IgG monoclonal conjugate. As seen in Table 15, when the polyclonal conjugate was used, the average was shifted from 0.0493 to 0.0931, and the standard deviation was increased from 0.074 to 0.0933. Moreover, the results also show that if the criteria of x +5SD is employed to define the assay cutoff, the polyclonal-enzyme conjugate configuration in the ELISA requires a higher cutoff value. This indicates a reduced assay specificity as compared to the monoclonal system. In addition, as depicted in the histogram in Fig. 44, a greater separation of results between negative and positive distributions occurs when random blood donors are screened in an ELISA using the anti-IgG monoclonal conjugate as compared to the assay using a commercial polyclonal label.

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TABLE 15

COMPARISON OF TWO ELISA CONFIGURATIONS IN

5	TESTING	SAMPLES	FROM	RANDOM	BLOOD	DONORS

10	CONJUGATE POL	YCLONAL	ANTI-IgG MONOCLONAL
	(Ja	ckson)	
	Number of samples	1073	1051
	Average (x)	0.0931	0.04926
• •	Standard deviation (SD)	0.0933	0.07427
15	5 SD	0.4666	0.3714
	CUT-OFF (5 SD + x)	0.5596	0.4206

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IV.J. Detection of HCV Seroconversion in NANBH Patients from a Variety of Geographical Locations

Sera from patients who were suspected to have

NANBH based upon elevated ALT levels, and who were
negative in HAV and HBV tests were screened using the RIA
essentially as described in Section IV.D., except that the
HCV C100-3 antigen was used as the screening antigen in
the microtiter plates. As seen from the results presented
in Table 16, the RIA detected positive samples in a high
percentage of the cases.

Table 16
Seroconversion Frequencies for Anti-c100-3

Among NANBH Patients in Different Countries

	Country	The Netherlands	<u>Italy</u>	Japan
20	No. Examined	5	36	26
	No. Positive	3	29	19
25	% Positive	60	80	73

IV.K. <u>Detection of HCV Seroconversion in Patients</u> with "Community Acquired" NANBH

Sera which was obtained from 100 patients with NANBH, for whom there was no obvious transmission route (i.e., no transfusions, i.v. drug use, promiscuity, etc. were identified as risk factors), was provided by Dr. M.

35 Alter of the Center for Disease Control, and Dr. J.

Dienstag of Harvard University. These samples were screened using an RIA essentially as described in Section IV.D., except that the HCV c100-3 antigen was used as the screening antigen attached to the microtiter plates. The results showed that of the 100 serum samples, 55 contained antibodies that reacted immunologically with the HCV c100-3 antigen.

The results described above suggest that

"Community Acquired" NANBH is also caused by HCV.

10 Moreover, since it has been demonstrated herein that HCV is related to Flaviviruses, most of which are transmitted by arthropods, it is suggestive that HCV transmission in the "Community Acquired" cases also results from arthropod transmission.

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IV.L. Comparison of Incidence of HCV Antibodies and Surrogate Markers in Donors Implicated in NANBH Transmission

A prospective study was carried out to determine whether recipients of blood from suspected NANBH positive 20 donors, who developed NANBH, seroconverted to anti-HCVantibody positive. The blood donors were tested for the surrogate marker abnormalities which are currently used as markers for NANBH infection, i.e., elevated ALT levels, and the presence of anti-core antibody. In addition, the 25 donors were also tested for the presence of anti-HCV antibodies. The determination of the presence of anti-HCV antibodies was determined using a radioimmunoassay as described in Section IV.K. The results of the study are 30 presented in Table 17, which shows: the patient number (column 1); the presence of anti-HCV antibodies in patient serum (column 2); the number of donations received by the patient, with each donation being from a different donor (column 3); the presence of anti-HCV antibodies in donor serum (column 4); and the surrogate abnormality of the

donor (column 5) (NT or -- means not tested) (ALT is elevated transaminase, and ANTI-HBc is anti-core antibody).

The results in Table 17 demonstrate that the HCV antibody test is more accurate in detecting infected blood donors than are the surrogate marker tests. Nine out of ten patients who developed NANBH symptoms tested positive for anti-HCV antibody seroconversion. Of the 11 suspected donors, (patient 6 received donations from two different individuals suspected of being NANBH carriers), 9 were positive for anti-HCV antibodies, and 1 was borderline positive, and therefore equivocal (donor for patient 1). In contrast, using the elevated ALT test 6 of the ten donors tested negative, and using the anticore-antibody 15 test 5 of the ten donors tested negative. Of greater consequence, though, in three cases (donors to patients 8, 9, and 10) the ALT test and the ANTI-HBc test yielded inconsistent results.

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		ຮູ	rogate rmality Anti-HB	ou	yes	ou	i i	yes	ou	yes	no	yes	ou	yes	
5		rs Carrief	Surrogate Abnormality Alt Anti-HB	Ou	IN	ou	;	yes	ou	yes	LN.	ou	yes	ou	
10		S IN PATIENTS BEING NANBH CARRIERS	Anti-HCV Positive Donor	equiv	yes	yes	ou	yes	yes(2)		yes	yes	yes	yes	
15	Table 17	HCV ANTIBODIE SUSPECTED OF	No. of Donations/Donors	18	18	13	18	. 16	11		15	20	5	15	Positive.
20		DEVELOPMENT OF ANTI- RECEIVING BLOOD FROM DONORS	Anti-HCV Seroconversion in Patient	хех	yes	yes	ou	yes	yes		yes	yes	yes	yes	or as anti-NANBV Positive.
25		I RECEIVING	Patient	7	7	m	4	S	9		7	ထ	თ	10	*Same donor
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IV.M. Amplification for Cloning of HCV cDNA Sequences Utilizing the PCR and Primers Derived from Conserved Regions of Flavivirus Genomic Sequences

The results presented supra., which suggest that 5 HCV is a flavivirus or flavi-like virus, allows a strategy for cloning uncharacterized HCV cDNA sequences utilizing the PCR technique, and primers derived from the regions encoding conserved amino acid sequences in flaviviruses. Generally, one of the primers is derived from a defined HCV genomic sequence, and the other primer which flanks a region of unsequenced HCV polynucleotide is derived from a conserved region of the flavivirus genome. The flavivirus genomes are known to contain conserved sequences within the NS1, and E polypeptides, which are encoded in the 5'region of the flavivirus genome. Corresponding sequences encoding these regions lie upstream of the HCV cDNA sequence shown in Fig. 26. Thus, to isolate cDNA sequences derived from this region of the HCV genome, upstream primers are designed which are derived from the conserved sequences within these flavivirus polypeptides. 20 The downstream primers are derived from an upstream end of the known portion of the HCV cDNA.

Because of the degeneracy of the code, it is probable that there will be mismatches between the flavivirus probes and the corresponding HCV genomic Therefore a strategy which is similar to the one described by Lee (1988) is used. The Lee procedure utilizes mixed oligonucleotide primers complementary to the reverse translation products of an amino acid sequence; the sequences in the mixed primers takes into account every codon degeneracy for the conserved amino acid sequence.

Three sets of primer mixes are generated, based on the amino acid homologies found in several flaviviruses, including Dengue-2,4 (D-2,4), Japanese

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Encephalitis Virus (JEV), Yellow Fever (YF), and West Nile Virus (WN). The primer mixture derived from the most upstream conserved sequence (5'-1), is based upon the amino acid sequence gly-trp-gly, which is part of the conserved sequence asp-arg-gly-trp-gly-aspN found in the E protein of D-2, JEV, YF, and WN. The next primer mixture (5'-2) is based upon a downstream conserved sequence in E protein, phe-asp-gly-asp-ser-tyr-ileu-phe-gly-asp-ser-tyrileu, and is derived from phe-gly-asp; the conserved sequence is present in D-2, JEV, YF, and WN. The third primer mixture (5'-3), is based on the amino acid sequence arg-ser-cys, which is part of the conserved sequence cyscys-arg-ser-cys in the NS1 protein of D-2, D-4, JEV, YF, and WN. The individual primers which form the mixture in 5'-3 are shown in Fig. 45. In addition to the varied sequences derived from conserved region, each primer in each mixture also contains a constant region at the 5'-end which contains a sequence encoding sites for restriction enzymes, HindIII, MboI, and EcoRI.

The downstream primer, ssc5h20A, is derived from a nucleotide sequence in clone 5h, which contains HCV cDNA with sequences with overlap those in clones 14i and 11b. The sequence of ssc5h20A is

5' GTA ATA TGG TGA CAG AGT CA 3'.

An alternative primer, ssc5h34A, may also be used. This primer is derived from a sequence in clone 5h, and in addition contains nucleotides at the 5'-end which create a restriction enzyme site, thus facilitating cloning. The sequence of ssc5h34A is

5' GAT CTC TAG AGA AAT CAA TAT GGT GAC AGA GTC A 3'.

The PCR reaction, which was initially described by Saiki et al. (1986), is carried out essentially as described in Lee et al. (1988), except that the template for the cDNA is RNA isolated from HCV infected chimpanzee liver, as described in Section IV.C.2., or from viral particles isolated from HCV infected chimpanzee serum, as described in Section IV.A.1. In addition, the annealing conditions are less stringent in the first round of amplification (0.6M NaCl, and 25°C), since the part of the primer which will anneal to the HCV sequence is only 9 nucleotides, and there could be mismatches. Moreover, if ssc5h34A is used, the additional sequences not derived from the HCV genome tend to destabilize the primertemplate hybrid. After the first round of amplification, the annealing conditions can be more stringent (0.066M NaCl, and 32°C-37°C), since the amplified sequences now contain regions which are complementary to, or duplicates of the primers. In addition, the first 10 cycles of amplification are run with Klenow enzyme I, under appropriate PCR conditions for that enzyme. After the com-20 pletion of these cycles, the samples are extracted, and run with Taq polymerase, according to kit directions, as furnished by Cetus/Perkin-Elmer.

After the amplification, the amplified HCV cDNA sequences are detected by hybridization using a probe derived from clone 5h. This probe is derived from sequences upstream of those used to derive the primer, and does not overlap the sequences of the clone 5h derived primers. The sequence of the probe is

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IV.N.1. Creation of HCV cDNA Library From Liver of a Chimpanzee with Infectious NANBH

An HCV cDNA library was created from liver from the chimpanzee from which the HCV cDNA library in Section IV.A.1. was created. The technique for creating the library was similar to that in Section IV.A.24, except for this different source of the RNA, and that a primer based on the sequence of HCV cDNA in clone 11b was used. The sequence of the primer was

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5' CTG GCT TGA AGA ATC 3'.

IV.N.2. <u>Isolation and Nucleotide Sequence of Overlapping</u> HCV cDNA in Clone k9-1 to cDNA in Clone 11b

Clone k9-1 was isolated from the HCV cDNA library created from the liver of an NANBH infected chimpanzee, as described in Section IV.A.25. The library was screened for clones which overlap the sequence in clone 11b, by using a clone which overlaps clone 11b at the 5'-terminus, clone 11e. The sequence of clone 11b is shown in Fig. 23. Positive clones were isolated with a frequency of 1 in 500,000. One isolated clone, k9-1, was subjected to further study. The overlapping nature of the HCV cDNA in clone k9-1 to the 5'-end of the HCV-cDNA sequence in Fig. 26 was confirmed by probing the clone with clone Alex46; this latter clone contains an HCV cDNA sequence of 30 base pairs which corresponds to those base pairs at the 5'terminus of the HCV cDNA in clone 14i, described supra.

The nucleotide sequence of the HCV cDNA isolated from clone k9-1 was determined using the techniques described supra. The sequence of the HCV cDNA in clone k9-1, the overlap with the HCV cDNA in Fig. 26, and the amino acids encoded therein are shown in Fig. 46.

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The HCV cDNA sequence in clone k9-1 has been aligned with those of the clones described in Section IV.A.19. to create a composite HCV cDNA sequence, with the k9-1 sequence being placed upstream of the sequence shown in Fig. 32. The composite HCV cDNA which includes the k9-1 sequence, and the amino acids encoded therein, is shown in Fig. 47.

The sequence of the amino acids encoded in the 5'-region of HCV cDNA shown in Fig. 47 has been compared with the corresponding region of one of the strains of Dengue virus, described supra., with respect to the profile of regions of hydrophobicity and hydrophilicity. This comparison showed that the polypeptides from HCV and Dengue encoded in this region, which corresponds to the region encoding NS1 (or a portion thereof), have a similar hydrophobic/hydrophilic profile.

The information provided infra. allows the identification of HCV strains. The isolation and characterization of other HCV strains may be accomplished by isolating the nucleic acids from body components which contain viral particles, creating cDNA libraries using polynucleotide probes based on the HCV cDNA probes described infra., screening the libraries for clones containing HCV cDNA sequences described infra., and comparing the HCV cDNAs from the new isolates with the cDNAs described infra. The polypeptides encoded therein, or in the viral genome, may be monitored for immunological cross-reactivity utilizing the polypeptides and antibodies described supra. Strains which fit within the parameters of HCV, as described in the Definitions section, supra., are readily identifiable. Other methods for identifying HCV strains will be obvious to those of skill in the art, based upon the information provided herein.

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Industrial Applicability

The invention, in the various manifestations disclosed herein, has many industrial uses, some of which are the following. The HCV cDNAs may be used for the design of probes for the detection of HCV nucleic acids in samples. The probes derived from the cDNAs may be used to detect HCV nucleic acids in, for example, chemical synthetic reactions. They may also be used in screening programs for anti-viral agents, to determine the effect of the agents in inhibiting viral replication in cell culture systems, and animal model systems. The HCV polynucleotide probes are also useful in detecting viral nucleic acids in humans, and thus, may serve as a basis for diagnosis of HCV infections in humans.

In addition to the above, the cDNAs provided herein provide information and a means for synthesizing polypeptides containing epitopes of HCV. These polypeptides are useful in detecting antibodies to HCV antigens. A series of immunoassays for HCV infection, based on recombinant polypeptides containing HCV epitopes are described herein, and will find commercial use in diagnosing HCV induced NANBH, in screening blood bank donors for HCV-caused infectious hepatitis, and also for detecting contaminated blood from infectious blood donors. The viral antigens will also have utility in monitoring the efficacy of anti-viral agents in animal model systems. In addition, the polypeptides derived from the HCV cDNAs disclosed herein will have utility as vaccines for treatment of HCV infections.

The polypeptides derived from the HCV cDNAs, besides the above stated uses, are also useful for raising anti-HCV antibodies. Thus, they may be used in anti-HCV vaccines. However, the antibodies produced as a result of immunization with the HCV polypeptides are also useful in detecting the presence of viral antigens in samples. Thus,

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they may be used to assay the production of HCV polypeptides in chemical systems. The anti-HCV antibodies may also be used to monitor the efficacy of anti-viral agents in screening programs where these agents are tested in tissue culture systems. They may also be used for passive immunotherapy, and to diagnose HCV caused NANBH by allowing the detection of viral antigen(s) in both blood donors and recipients. Another important use for anti-HCV antibodies is in affinity chromatography for the purification of virus and viral polypeptides. 10 purified virus and viral polypeptide preparations may be used in vaccines. However, the purified virus may also be useful for the development of cell culture systems in which HCV replicates.

Cell culture systems containing HCV infected cells will have many uses. They can be used for the relatively large scale production of HCV, which is normally a low titer virus. These systems will also be useful for an elucidation of the molecular biology of the 20 virus, and lead to the development of anti-viral agents. The cell culture systems will also be useful in screening for the efficacy of antiviral agents. In addition, HCV permissive cell culture systems are useful for the production of attenuated strains of HCV.

For convenience, the anti-HCV antibodies and HCV polypeptides, whether natural or recombinant, may be packaged into kits.

The method used for isolating HCV cDNA, which is comprised of preapring a cDNA library derived from infected tissue of an individual, in an expression vector, and selecting clones which produce the expression products which react immunologically with antibodies in antibodycontaining body components from other infected individuals and not from non-infected individuals, may also be applicable to the isolation of cDNAs derived from other

heretofore uncharacterized disease-associated agents which are comprised of a genomic component. This, in turn, could lead to isolation and characterization of these agents, and to diagnostic reagents and vaccines for these other disease-associated agents.

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CLAIMS

- 1. A purified HCV polynucleotide.
- 5 2. A recombinant HCV polynucleotide.
 - 3. A recombinant polynucleotide comprising a sequence derived from an HCV genome or from HCV cDNA.
- 10 4. A recombinant polynucleotide encoding an epitope of HCV.
 - 5. A recombinant vector containing the polynucleotide of claim 2, or claim 3, or claim 4.
 - 6. A host cell transformed with the vector of claim 5.
- 7. A recombinant expression system comprising 20 an open reading frame (ORF) of DNA derived from an HCV genome or from HCV cDNA, wherein the ORF is operably linked to a control sequence compatible with a desired host.
- 25 8. A cell transformed with the recombinant expression system of claim 7.
 - A polypeptide produced by the cell of claim
 - 10. Purified HCV.
 - 11. A preparation of polypeptides from the (HCV of claim 10.

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- 12. A purified HCV polypeptide.
- 13. A purified polypeptide comprising an epitope which is immunologically identifiable with an epitope contained in HCV.
 - 14. A recombinant HCV polypeptide.
- 15. A recombinant polypeptide comprised of a sequence derived from an HCV genome or from HCV cDNA.
 - 16. A recombinant polypeptide comprised of an HCV epitope.
- 15 17. A fusion polypeptide comprised of an HCV polypeptide.
 - 18. A monoclonal antibody directed against an HCV epitope.
 - 19. A purified preparation of polyclonal antibodies directed against HCV.
- 20. A particle which is immunogenic against HCV infection comprising a non-HCV polypeptide having an amino acid sequence capable of forming a particle when said sequence is produced in a eukaryotic host, and an HCV epitope.
- 30 21. A polynucleotide probe for HCV.
 - 22. A kit for analyzing samples for the presence of polynucleotides derived from HCV comprising a polynucleotide probe containing a nucleotide sequence from

HCV of about 8 or more nucleotides, in a suitable container.

- 23. A kit for analyzing samples for the presence of an HCV antigen comprising an antibody directed against the HCV antigen to be detected, in a suitable container.
- 24. A kit for analyzing samples for the 10 presence of an antibodies directed against an HCV antigen comprising a polypeptide containing an HCV epitope present in the HCV antigen, in a suitable container.
- 25. A polypeptide comprised of an HCV epitope, 15 attached to a solid substrate.
 - 26. An antibody to an HCV epitope, attached to a solid substrate.
- 27. A method for producing a polypeptide containing an HCV epitope comprising incubating host cells transformed with an expression vector containing a sequence encoding a polypeptide containing an HCV epitope under conditions which allow expression of said polypeptide.
 - 28. A polypeptide containing an HCV epitope produced by the method of claim 27.
- 30 29. A method for detecting HCV nucleic acids in a sample comprising:
- (a) reacting nucleic acids of the sample with a probe for an HCV polynucleotide under conditions which allow the formation of a polynucleotide duplex between the probe and the HCV nucleic acid from the sample; and

- --, --- .

- (b) detecting a polynucleotide duplex which contains the probe.
- 30. An immunoassay for detecting an HCV antigen comprising:
 - (a) incubating a sample suspected of containing an HCV antigen with a probe antibody directed against the HCV antigen to be detected under conditions which allow the formation of an antigen-antibody complex; and
- 10 (b) detecting an antigen-antibody complex containing the probe antibody.
 - 31. An immunoassay for detecting antibodies directed against an HCV antigen comprising:
- (a) incubating a sample suspected of containing anti-HCV antibodies with a probe polypeptide which contains an epitope of the HCV, under conditions which allow the formation of an antibody-antigen complex; and
- (b) detecting the antibody-antigen complex containing the probe antigen.
 - 32. A vaccine for treatment of HCV infection comprising an immunogenic polypeptide containing an HCV epitope wherein the immunogenic polypeptide is present in a pharmacologically effective dose in a pharmaceutically acceptable excipient.
- 33. A vaccine for treatment of HCV infection comprising inactivated HCV in a pharmacologically effective dose in a pharmaceutically acceptable excipient.
 - 34. A vaccine for treatment of HCV infection comprising attenuated HCV in a pharmacologically effective dose in a pharmaceutically acceptable excipient.

- 35. A tissue culture grown cell infected with HCV.
- 36. The HCV infected cell of claim 35, wherein the cell is of a human macrophage cell line, or is of a hepatocyte cell line, or is of a mosquito cell line, or is of a tick cell line, or is of a mouse macrophage cell line, or is an embryonic cell.
- 10 37. The HCV infected cell of claim 35, wherein the cell is of a cell line derived from liver of an HCV infected individual.
- 38. A method for producing antibodies to HCV comprising administering to an individual an isolated immunogenic polypeptide containing an HCV epitope in an amount sufficient to produce an immune response.
- 39. A method for producing antibodies to HCV comprising administering to an individual the polypeptide preparation of claim 11, wherein the preparation contains at least 1 immunogenic polypeptide, and the administering is of an amount sufficient to produce an immune response.
- 25 40. A method for isolating cDNA derived from the genome of an unidentified infectious agent, comprising:
 - (a) providing host cells transformed with expression vectors containing a cDNA library prepared from nucleic acids isolated from tissue infected with the agent and growing said host cells under conditions which allow expression of polypeptide(s) encoded in the cDNA;
- (b) interacting the expression products of the cDNA with an antibody containing body component of an individual infected with said infectious agent under

conditions which allow an immunoreaction, and detecting antibody-antigen complexes formed as a result of the interacting;

- (c) growing host cells which expresspolypeptides that form antibody-antigen complexes in step(b) under conditions which allow their growth asindividual clones and isolating said clones;
- (d) growing cells from the clones of (c) under conditions which allow expression of polypeptide(s)
 10 encoded within the cDNA, and interacting the expression products with antibody containing body components of individuals other than the individual in step (a) who are infected with the infectious agent and with control individuals uninfected with the agent, and detecting
 15 antibody-antigen complexes formed as a result of the interacting;
 - (e) growing host cells which express polypeptides that form antibody-antigen complexes with antibody containing body components of infected individuals and individuals suspected of being infected, and not with said components of control individuals, under conditions which allow their growth as individual clones and isolating said clones; and
- $% \left(1\right) =\left(1\right) \left(1\right) =\left(1\right) \left(1\right)$ (f) isolating the cDNA from the host cell clones of (e).

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FIG | Translation of DNA 5-1-1

- AlaSerCysLeuAsnCysSerAlaSerIleIleProAspArgGluValLeuTyrArgGlu

 1 GGCCTCCTGCTTGAACTGCTCGGCGAGCATCATACCTGACAGGGAAGTCCTCTACCGAGA
 CCGGAGGACGAACTTGACGAGCCGCTCGTAGTATGGACTGTCCCTTCAGGAGATGGCTCT
- PheAspGluMetGluGluCysSerGlnHisLeuProTyrIleGluGlnGlyMetMetLeu
 61 GTTCGATGAGATGGAAGAGTGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCT
 CAAGCTACTCTACCTTCTCACGAGAGTCGTGAATGGCATGTAGCTCGTTCCCTACTACGA
- AlaGluGlnPheLysGlnLysAlaLeuGlyLeu 121 CGCCGAGCAGTTCAAGCAGAAGGCCCTCGGCCTCC GCGGCTCGTCAAGTTCGTCTTCCGGGAGCCGGAGG

FIG. 3 Translation of DNA 5-1-1,81,91&1-2

- GlyCysValValIleValGlyArgValValLeuSerGlyLysProAlaIleIleProAsp 1 CTGGCTGCGTGGTCATAGTGGGCAGGGTCGTCTTGTCCGGGAAGCCGGCAATCATACCTG GACCGACGCACCAGTATCACCCGTCCCAGCAGAACAGGCCCTTCGGCCGTTAGTATGGAC
- ArgGluValLeuTyrArgGluPheAspGluMetGluGluCysSerGlnHisLeuProTyr
 ACAGGGAAGTCCTCTACCGAGAGTTCGATGAGATGGAAGAGTGCTCTCAGCACTTACCGT
 TGTCCCTTCAGGAGATGGCTCTCAAGCTACCTTCTCACGAGAGTCGTGAATGGCA
 A
- IleGluGlnGlyMetMetLeuAlaGluGlnPheLysGlnLysAlaLeuGlyLeuLeuGln
 121 ACATCGAGCAAGGGATGATGCTCGCCGAGCAGTTCAAGCAGAAGGCCCTCGGCCTCCTGC
 TGTAGCTCGTTCCCTACTACGAGCGGCTCGTCAAGTTCGTCTTCCGGGAGCCGGAGGACG
- ThrAlaSerArgGlnAlaGluValIleAlaProAlaVálGlnThrAsnTrpGlnLysLeu
 181 AGACCGCGTCCCGTCAGGCAGAGGTTATCGCCCCTGCTGTCCAGACCAACTGGCAAAAAC
 TCTGGCGCAGGGCAGTCCGTCTCCAATAGCGGGGACGACAGGTCTGGTTGACCGTTTTTG
- GluThrPheTrpAlaLysHisMetTrpAsnPheIleSerGlyIleGlnTyrLeuAlaGly
 TCGAGACCTTCTGGGCGAAGCATATGTGGAACTTCATCAGTGGGATACAATACTTGGCGG
 AGCTCTGGAAGACCCGCTTCGTATACACCTTGAAGTAGTCACCCTATGTTATGAACCGCC
- LeuSerThrLeuProGlyAsnProAlaIleAlaSerLeuMetAlaPheThrAlaAlaVal
 GCTTGTCAACGCTGCCTGGTAACCCCGCCATTGCTTCATTGATGGCTTTTACAGCTGCTG
 CGAACAGTTGCGACGGACCATTGGGGCGGTAACGAAGTAACTACCGAAAATGTCGACGAC
- ThrserProLeuThrThrSerGln
 361 TCACCAGCCCACTAACCACTAGCCAAA
 AGTGGTCGGGTGATTGGTGATCGGTTT

China

5-1-1	-	[ggcctcctgcttgaactgctcggcgagc]
18	7	GTCCGGGAAGCCGCCATCATACCTGACAGGGAAG
16	1	1 ctggctgcgtggtcatagrggccaggtrcfrcfrccgggaagccggcarcatactrgacaggaag
1-2	7	GGICATAGTGGGCAGGGTCGTTTGTCCGGGAAGCCGGCAATCATACCTGACAGGGAAG
<u> - - 2</u>	48	TCTCTACCGAGAGTTCGATGAATGGAAGAGTGCTCTCAGCACTTACCGTACATCGAGGGATGATGC
10	36	=5 =2
91	70	1 CCTCTACCGAGAGTTCGATGGAAGGGAGGGAGGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGC
1-2	09	111111 1111111111111111111111111111111
5-1-1	120	TCGCCGAGCAGTTCAAGCAGAAGGCCCTTCC
10	108	-
16	142	TCGCCGAGCAGTTCAAGCAGAAGGCCCTCGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGGTTATCGCCC
1-3	132	TCCCCGAGCAGTTCAAGCAGAAGGCCCTCGGCC
18	180	CTGCTICTCCAGACCAACTIGGCAAAAACTICGAGACCTTCTIGGGCGAAGCATATGTGGAACTTTCAGTGGGA
91	214	CIGCIGITOTION CONTROLLE CINCOLO CONTROLLA CONTR
81	252	TACAATACTIGGCGGGCTTGTCAACGCTGCCTGGtaaccccgccattgcttcattgatggctttacagctg
91	286	THEFTER THE TELEVISION OF THE T
	•	מ טוי
18	32,	334 ctgtcaccagccactagccaaa

FIG. 4 Translation of DNA 81

- SerGlyLysProAlaIleIleProAspArgGluValLeuTyrArgGluPheAspGluMet 1 GTCCGGGAAGCCGGCAATCATACCTGACAGGGAAGTCCTCTACCGAGAGTTCGATGAGAT CAGGCCCTTCGGCCGTTAGTATGGACTGTCCCTTCAGGAGATGGCTCTCAAGCTACTCTA
- GluGluCysSerGlnHisLeuProTyrIleGluGlnGlyMetMetLeuAlaGluGlnPhe 61 GGAAGAGTGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCTCGCCGAGCAGTT CCTTCTCACGAGAGTCGTGAATGGCATGTAGCTCGTTCCCTACTACGAGCGGCTCGTCAA
- LysGlnLysAlaLeuGlyLeuLeuGlnThrAlaSerArgGlnAlaGluValIleAlaPro
 CAAGCAGAAGGCCCTCGGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGGTTATCGCCCC
 GTTCGTCTTCCGGGAGCCCGGAGGACGTCTGGCGCAGGGCAGTCCGTCTCCAATAGCGGGG
- AlaValGlnThrAsnTrpGlnLysLeuGluThrPheTrpAlaLysHisMetTrpAsnPhe
 181 TGCTGTCCAGACCAACTGGCAAAAACTCGAGACCTTCTGGGCGAAGCATATGTGGAACTT
 ACGACAGGTCTGGTTGACCGTTTTTGAGCTCTGGAAGACCCGCTTCGTATACACCTTGAA
- IleSerGlyIleGlnTyrLeuAlaGlyLeuSerThrLeuProGlyAsnProAlaIleAla 241 CATCAGTGGGATACATACTTGGCGGGCTTGTCAACGCTGCCTGGTAACCCCCGCCATTGC GTAGTCACCCTATGTTATGAACCGCCCGAACAGTTGCGACGACCATTGGGGCGGTAACG
- SerLeuMetAlaPheThrAlaAlaValThrSerProLeuThrThrSerGln
 TTCATTGATGGCTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCCAAA
 AAGTAACTACCGAAAATGTCGACGACAGTGGTCGGGTGATTGGTGATCGGTTT

FIG. 5 Translation of DNA 36

- TyrGlnAlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAspGlnMetTrp

 1ACCAAGCCACCGTGTGCGCTAGGGCTCAAGCCCCTCCCCCATCGTGGGACCAGATGTGG

 ATGGTTCGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGGTAGCACCCTGGTCTACACC
- LysCysLeuIleArgLeuLysProThrLeuHisGlyProThrProLeuLeuTyrArgLeu

 121 AAGTGTTTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGCTATACAGACTG

 TTCACAAACTAAGCGGAGTTCGGGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGAC
- GlyAlaValGlnAsnGluIleThrLeuThrHisProValThrLysTyrIleMetThrCys GGCGCTGTTCAGAATGAAATCACCCTGACGCACCCAGTCACCAAATACATCATGACATGC CCGCGACAAGTCTTACTTTAGTGGGACTGCGTGGGTCAGTGGTTTATGTAGTACTGTACG
- MetSerAlaAspLeuGluValValThrSerThrTrpValLeuValGlyGlyValLeuAla 241 ATGTCGGCCGACCTGGAGGTCGTCACGAGCACCTGGGTGCTCGTTGGCGGCGTCCTGGCT TACAGCCGGCTGGACCTCCAGCAGTGCTCGTGGACCCACGAGCAACCGCCGCAGGACCGA
- AlaLeuAlaAlaTyrCysLeuSerThrGlyCysValValIleValGlyArgValValLeu
 301 GCTTTGGCCGCGTATTGCCTGTCAACAGGCTGCGTCATAGTGGGCAGGGTCGTCTTG
 CGAAACCGGCGCATAACGGACAGTTGTCCGACGCACCAGTATCACCCGTCCCAGCAGAAC

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FIG. 6 Combined ORF of DNAs 36 & 81

- TyrGlnAlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAspGlnMetTrp
 61 TACCAAGCCACCGTGTGCGCTAGGGCTCAAGCCCCCCATCGTGGGACCAGATGTGG
 ATGGTTCGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGGGTAGCACCCTGGTCTACACC
- LysCysLeulleArgLeuLysProThrLeuHisGlyProThrProLeuLeuTyrArgLeu
 121 AAGTGTTTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGCTATACAGACTG
 TTCACAAACTAAGCGGAGGTTCGGGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGAC
- GlyAlaValGlnAsnGluIleThrLeuThrHisProValThrLysTyrIleMetThrCys GGCGCTGTTCAGAATGAAATCACCCTGACGCACCCAGTCACCAAATACATCATGACATGC CCGCGACAAGTCTTACTTTAGTGGGACTGCGTGGGTCAGTGGTTTATGTAGTACTGTACG
- AlaLeuAlaAlaTyrCysLeuSerThrGlyCysValVallleValGlyArgValValLeu
 301 GCTTTGGCCGCGTATTGCCTGTCAACAGGCTGCGTCGTCATAGTGGGCAGGGTCGTCTTG
 CGAAACCGGCGCATAACGGACAGTTGTCCGACGCACCAGTATCACCCGTCCCAGCAGAAC
- SerGlyLysProAlaIleIleProAspArgGluValLeuTyrArgGluPheAspGluMet
 TCCGGGAAGCCGGCAATCATACCTGACAGGGAAGTCCTCTACCGAGAGTTCGATGAGATG
 AGGCCCTTCGGCCGTTAGTATGGACTGTCCCTTCAGGAGATGGCTCTCAAGCTACTCTAC
- GluGluCysSerGlnHisLeuProTyrIleGluGlnGlyMetMetLeuAlaGluGlnPhe GAAGAGTGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCTCGCCGAGCAGTTC CTTCTCACGAGAGTCGTGAATGGCATGTAGCTCCTACTACGAGCGGCTCGTCAAG
- LysGlnLysAlaLeuGlyLeuLeuGlnThrAlaSerArgGlnAlaGluValIleAlaPro
 481 AAGCAGAAGGCCCTCGGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGGTTATCGCCCCT
 TTCGTCTTCCGGGAGCCGGAGGACGTCTGGCGCAGGGCAGTCCGTCTCCAATAGCGGGGA
- AlaValGlnThrAsnTrpGlnLysLeuGluThrPheTrpAlaLysHisMetTrpAsnPhe
 541 GCTGTCCAGACCAACTGGCAAAAACTCGAGACCTTCTGGGCGAAGCATATGTGGAACTTC
 CGACAGGTCTGGTTGACCCTTTTTGAGCTCTGGAAGACCCGCTTCGTATACACCTTGAAG
- IleSerGlyIleGlnTyrLeuAlaGlyLeuSerThrLeuProGlyAsnProAlaIleAla
 ATCAGTGGGATACAATACTTGGCGGGCTTGTCAACGCTGCCTGGTAACCCCGCCATTGCT
 TAGTCACCCTATGTTATGAACCGCCCGAACAGTTGCGACGGACCATTGGGGCGGTAACGA
- SerLeuMetAlaPheThrAlaAlaValThrSerProLeuThrThrSerGln
 TCATTGATGGCTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCCAAA
 AGTAACTACCGAAAATGTCGACGACAGTGGTCGGTTGATTGGTGATCGGTTT

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FIG. 7 Translation of DNA 32

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- ----Overlap with 81----PheThrAlaAlaValThrSerProLeuThrThrSerGlnThrLeuLeuPheAsnIleLeu
 CTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCCAAACCCTCCTCTTCAACATAT
 GAAAATGTCGACGACAGTGGTCGGGTGATTGGTGATCGGTTTGGGAGGAGAAGTTGTATA
- GlyGlyTrpValAlaAlaGlnLeuAlaAlaProGlyAlaAlaThrAlaPheValGlyAla

 1 TGGGGGGGTGGGTGCCCAGCTCGCCCCCCGGTGCCGCTACTGCCTTTGTGGGCG
 ACCCCCCACCCACCGACGGGTCGAGCGGGGGGCCACGGCGATGACGGAAACACCCGC
- GlyLeuAlaGlyAlaAlaIleGlySerValGlyLeuGlyLysValLeuIleAspIleLeu
 121 CTGGCTTAGCTGGCGCCGCCATCGGCAGTGTTGGACTGGGGAAGGTCCTCATAGACATCC
 GACCGAATCGACCGCGGCGGTAGCCGTCACAACCTGACCCCTTCCAGGAGTATCTGTAGG
- AlaGlyTyrGlyAlaGlyValAlaGlyAlaLeuValAlaPheLysIleMetSerGlyGlu 181 TTGCAGGGTATGGCGCGGGCGTGGCGGAGCTCTTGTGGCATTCAAGATCATGAGCGGTG AACGTCCCATACCGCCCCGCACCGCCCTCGAGAACACCGTAAGTTCTAGTACTCGCCAC
- ValProSerThrGluAspLeuValAsnLeuLeuProAlaIleLeuSerProGlyAlaLeu
 241 AGGTCCCCTCCACGGAGGACCTGGTCAATCTACTGCCCGCCATCCTCTCGCCCGGAGCCC
 TCCAGGGGAGGTGCCTCCTGGACCAGTTAGATGACGGGCGGTAGGAGAGCGGGCCTCGGG
- ValValGlyValValCysAlaAlaIleLeuArgArgHisValGlyProGlyGluGlyAla TCGTAGTCGGCGTGGTCTGTGCAGCAATACTGCGCCGCACGTTGGCCCGGGCGAGGGGG AGCATCAGCCGCACCAGACACGTCGTTATGACGCGGCCGTGCAACCGGGCCCGCTCCCCC

FIG. 8 Translation of DNA 35

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- SerileGluThrIleThrLeuProGlnAspAlaValSerArgThrGlnArgArgGlyArg 1 TCCATTGAGACAATCACGCTCCCCCAGGATGCTGTCTCCCGCACTCAACGTCGGGGCAGG AGGTAACTCTGTTAGTGCGAGGGGGTCCTACGACAGAGGGCGTGAGTTGCAGCCCCGTCC
- ThrGlyArgGlyLysProGlyIleTyrArgPheValAlaProGlyGluArgProSerGly
 61 ACTGGCAGGGGAAGCCAGGCATCTACAGATTTGTGGCACCGGGGGAGCCCCCTCCGGC
 TGACCGTCCCCCTTCGGTCCGTAGATGTCTAAACACCGTGGCCCCCTCGCGGGGAGGCCG
- MetPheAspSerSerValLeuCysGluCysTyrAspAlaGlyCysAlaTrpTyrGluLeu
 121 ATGTTCGACTCCGTCCTCTGTGAGTGCTATGACGCAGGCTGTGCTTGGTATGAGCTC
 TACAAGCTGAGCAGGCAGGAGACACTCACGATACTGCGTCCGACACGAACCATACTCGAG
- ThrProAlaGluThrThrValArgLeuArgAlaTyrMetAsnThrProGlyLeuProVal
 ACGCCCGCCGAGACTACAGTTAGGCTACGAGCGTACATGAACACCCCGGGGCTTCCCGTG
 TGCGGGCGGCTCTGATGTCAATCCGATGCTCGCATGTACTTGTGGGGCCCCCGAAGGGCAC

- AlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAspGlnMetTrpLysCys
 GCCACCGTGTGCGCTAGGGCTCAAGCCCCTCCCCCATCGTGGGACCAGATGTGGAAGTGT
 CGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGTAGCACCCTTCACAC
- LeulleArgLeuLysProThrLeuHisGlyProThrProLeuLeuTyrArgLeuGlyAla
 TTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGCTATACAGACTGGGCGCT
 AACTAAGCGGAGTTCGGGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGACCCGCGA

CHDCTITHE CHFFT

FIG. 9-1 Combined ORF of DNAs 35,36,81 & 32

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- SerileGluthrileThrLeuProGlnAspAlaValSerArgThrGlnArgArgGlyArg 1 TCCATTGAGACAATCACGCTCCCCCAGGATGCTGTCTCCCGCACCTCAACGTCGGGGCAGG AGGTAACTCTGTTAGTGCGAGGGGGTCCTACGACAGAGGGCGTGAGTTGCAGCCCCGTCC
- ThrGlyArgGlyLysProGlyIleTyrArgPheValAlaProGlyGluArgProSerGly
 61 ACTGGCAGGGGAAGCCAGGCATCTACAGATTTGTGGCACCGGGGGAGCGCCCCTCCGGC
 TGACCGTCCCCCTTCGGTCCGTAGATGTCTAAACACCGTGGCCCCCTTCGCGGGGAGGCCG
- MetPheAspSerSerValLeuCysGluCysTyrAspAlaGlyCysAlaTrpTyrGluLeu
 121 ATGTTCGACTCGTCCGTCCTCTGTGAGTGCTATGAGCGCAGGCTGTGCTTGGTATGAGCTC
 TACAAGCTGAGCAGGCAGGAGACACTCACGATACTGCGTCCGACACGAACCATACTCGAG
- ThrProAlaGluThrThrValArgLeuArgAlaTyrMetAsnThrProGlyLeuProVal
 ACGCCCGCCGAGACTACAGTTAGGCTACGAGCGTACATGAACACCCCGGGGCTTCCCGTG
 TGCGGGCGGCTCTGATGTCAATCCGATGCTCGCATGTACTTGTGGGGCCCCCGAAGGGCAC

- AlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAspGlnMetTrpLysCys
 GCCACCGTGTGCGCTAGGGCTCAAGCCCCTCCCCCATCGTGGGACCAGATGTGGAAGTGT
 CGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGGTAGCACCCTGGTCTACACCTTCACA
- LeulleArgLeuLysProThrLeuHisGlyProThrProLeuLeuTyrArgLeuGlyAla
 TTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGCTATACAGACTGGGCGCT
 AACTAAGCGGAGTTCGGGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGACCCGCGA
- AlaAspLeuGluValValThrSerThrTrpValLeuValGlyGlyValLeuAlaAlaLeu 541 GCCGACCTGGAGGTCGTCACGAGCACCTGGGTGCTCGTTGGCGGCGTCCTGGCTTTG CGGCTGGACCTCCAGCAGTGCTCGTGGACCCACGAGCAACCGCCGCAGGACCGACGAAAC
- AlaAlaTyrCysLeuSerThrGlyCysValValIleValGlyArgValValLeuSerGly
 GCCGCGTATTGCCTGTCAACAGGCTGCGTCATAGTGGGCAGGGTCGTCTTGTCCGGG
 CGGCGCATAACGGACAGTTGTCCGACGCACCAGTATCACCCGTCCCAGCAGAACAGGCCC
- LysProAlaIleIleProAspArgGluValLeuTyrArgGluPheAspGluMetGluGlu
 661 AAGCCGGCAATCATACCTGACAGGGAAGTCCTCTACCGAGAGTTCGATGAGATGGAAGAG
 TTCGGCCGTTAGTATGGACTGTCCCTTCAGGAGATGGCTCTCAAGCTACTCTACCTTCTC
- CysSerGlnHisLeuProTyrlleGluGlnGlyMetMetLeuAlaGluGlnPheLysGln
 TGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCTCGCCGAGCAGTTCAAGCAG
 ACGAGAGTCGTGAATGGCATGTAGCTCGTTCCCTACTACGAGCGGCTCGTCAAGTTCGTC
- LysalaLeuGlyLeuLeuGlnThralaSerArgGlnAlaGluVallleAlaProAlaVal
 781 AAGGCCTCGGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGGTTATCGCCCCTGCTGTC
 TTCCGGGAGCCGGAGGACGTCTGGCGCAGGGCAGTCCGTCTCCAATAGCGGGGACGACAG

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	GlnThrAsnTrpGlnLysLeuGluThrPheTrpAlaLysHisMetTrpAsnPheIleSer
943	CACACCA ACTGGCAAAAACTCGAGACCTTCTGGGCGAAGCATATGTGGAACTTCATCAGT
	GTCTGGTTGACCGTTTTTGAGCTCTGGAAGACCCGCTTCGTATACACCTTGAAGTAGTCA

- GlylleGlnTyrLeuAlaGlyLeuSerThrLeuProGlyAsnProAlaIleAlaSerLeu
 GGGATACAATACTTGGCGGGCTTGTCAACGCTGCCTGGTAACCCCGCCATTGCTTCATTG
 CCCTATGTTATGAACCGCCCGAACAGTTGCGACGACCATTGGGGGCGGTAACGAAGTAAC
- MetalaPheThrAlaAlaValThrSerProLeuThrThrSerGlnThrLeuLeuPheAsn 961 ATGGCTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCCAAACCCTCCTCTTCAAC TACCGAAAATGTCGACGACAGTGGTCGGGTGATTGGTGATCGGTTTTGGGAGGAGAAGTTG
- IleLeuGlyGlyTrpValAlaAlaGlnLeuAlaAlaProGlyAlaAlaThrAlaPheVal
 ATATTGGGGGGGTGGCTGCCCAGCTCGCCGCCCCGGTGCCGCTACTGCCTTTGTG
 TATAACCCCCCCACCGACGGGTCGAGCGGGGGGCCACGGCGATGACGGAAACAC
- GlyAlaGlyLeuAlaGlyAlaAlaIleGlySerValGlyLeuGlyLysValLeuIleAsp GGCGCTGGCTTAGCTGGCGCCGCCATCGGCAGTGTTGGACTGGGGAAGGTCCTCATAGAC CCGCGACCGAATCGACCGCGGCGGTAGCCGTCACAACCTGACCCCTTCCAGGAGTATCTG
- IleLeuAlaGlyTyrGlyAlaGlyValAlaGlyAlaLeuValAlaPheLysIleMetSer ATCCTTGCAGGGTATGGCGCGGGCGTGGCGGAGCTCTTGTGGCATTCAAGATCATGAGC TAGGAACGTCCCATACCGCGCCCGCACCGCCCTCGAGAACACCGTAAGTTCTAGTACTCG
- GlyGluValProSerThrGluAspLeuValAsnLeuLeuProAlaIleLeuSerProGly
 1201 GGTGAGGTCCCCTCCACGGAGGACCTGGTCAATCTACTGCCCGCCATCCTCTCGCCCGGA
 CCACTCCAGGGGAGGTGCCTCCTGGACCAGTTAGATGACGGGCGGTAGGAGAGCGGGCCT
- AlaLeuValValGlyValValCysAlaAlaIleLeuArgArgHisValGlyProGlyGlu
 1261 GCCCTCGTAGTCGGCGTGGTCTGTGCAGCAATACTGCGCCGGCACGTTGGCCCGGGCGAG
 CGGGAGCATCAGCCGCACCAGACACGTCGTTATGACGCGGCCGTGCAACCGGGCCCGCTC

FIG. 9-2

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FIG. 10 Translation of DNA 37b

- LeuAlaAlaLysLeuValAlaLeuGlyIleAsnAlaValAlaTyrTyrArgGlyLeuAsp 1 CTCGCCGCAAAGCTGGTCGCATTGGGCATCAATGCCGTGGCCTACCACCGCGTCTTGAC GAGCGGCGTTTCGACCAGCGTAACCCGTAGTTACGGCACCGGATGATGGCGCCAGAACTG
- ValSerValIleProThrSerGlyAspValValValAlaThrAspAlaLeuMetThr 61 GTGTCCGTCATCCCGACCAGCGGCGATGTTGTCGTCGTCGCCAACCGATGCCCTCATGACC CACAGGCAGTAGGGCTGGTCGCCGCTACAACAGCAGCACCGTTGGCTACGGGAGTACTGG
- GlyTyrThrGlyAspPheAspSerValIleAspTyrAsnThrCysValThrGlnThrVal 121 GGCTATACCGGCGACTTCGACTCGGTGATAGACTACAATACGTGTGTCACCCCAGACAGTC CCGATATGGCCGCTGAAGCTGAGCCACTATCTGATGTTATGCACACAGTGGGTCTGTCAG
- AspPheSerLeuAspProThrPheThrIleGluThrIleThrLeuProGlnAspAlaVal

 181 GATTTCAGCCTTGACCCTTCACCATTGAGACAATCACGCTCCCCCAGGATGCTGTC
 CTAAAGTCGGAACTGGGATGGAAGTGGTAACTCTGTTAGTGCGAGGGGGTCCTACGACAG

FIG. | Translation of DNA 33b

- ProGluSerAspAlaAlaArgValThrAlaIleLeuSerSerLeuThrValThrGln
 61 GCCGGAGAGCGATGCAGCTGCCCGCGTCACTGCAACTCAGCAGCCTCACTGTAACCCA
 CGGCCTCTCGCTACGTCGACGGCGCAGTGACGGTATGAGTCGTCGGAGTGACATTGGGT
- LeuLeuArgArgLeuHisGlnTrplleSerSerGluCysThrThrProCysSerGlySer
 121 GCTCCTGAGGCGACTGCACCAGTGGATAAGCTCGGAGTGTACCACTCCATGCTCCGGTTC
 CGAGGACTCCGCTGACGTGGTCACCTATTCGAGCCTCACATGGTGAGGTACGAGGCCAAG
- TrpLeuArgAspIleTrpAspTrpIleCysGluValLeuSerAspPheLysThrTrpLeu
 181 CTGGCTAAGGGACATCTGGGACTGGATATGCGAGGTGTTGAGCGACTTTAAGACCTGGCT
 GACCGATTCCCTGTAGACCCTGACCTATACGCTCCACAACTCGCTGAAATTCTGGACCGA
- LysGlyValTrpArgVal
 301 TAAGGGGGTCTGGCGAGTG
 ATTCCCCCAGACCGCTCAC

CUPATITUTE CUEET

FIG 12 Translation of DNA 40b

- AlaTyrMetSerLysAlaHisGlyIleAspProAsnIleArgThrGlyValArgThrIle
 GGCTTACATGTCCAAGGCTCATGGGATCGATCCTAACATCAGGACCGGGGTGAGAACAAT
 CCGAATGTACAGGTTCCGAGTACCCTAGCTAGGATTGTAGTCCTGGCCCCACTCTTGTTA
- ThrThrGlySerProlleThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGlyCys

 TACCACTGGCAGCCCCATCACGTACTCCACCTACGGCAAGTTCCTTGCCGACGGCGGTG

 ATGGTGACCGTCGGGGTAGTGCATGAGGTGGATGCCGTTCAAGGAACGGCTGCCGCCCAC
- SerGlyGlyAlaTyrAspIleIleIleCysAspGluCysHisSerThrAspAlaThrSer
 121 CTCGGGGGGCGCTTATGACATAATAATTTGTGACGAGTGCCACTCCACGGATGCCACATC
 GAGCCCCCCGCGAATACTGTATTATAAACACTGCTCACGGTGAGGTGCCTACGGTGTAG
- IleLeuGlyIleGlyThrValLeuAspGlnAlaGluThrAlaGlyAlaArgLeuValVal

 181 CATCTTGGGCATCGGCACTGTCCTTGACCAAGCAGAGACTGCGGGGGCGAGACTGGTTGT
 GTAGAACCCGTAGCCGTGACAGGAACTGGTTCGTCTCTGACGCCCCCGCTCTGACCAACA
- LeuAlaThrAlaThrProProGlySerValThrValProHisProAsnIleGluGluVal
 241 GCTCGCCACCGCCACCCCTCCGGGCTCCGTCACTGTGCCCCATCCCAACATCGAGGAGGT
 CGAGCGGTGGCGGTGGGGAGGCCCGAGGCAGTGACACGGGGTAGGGTTGTAGCTCCTCCA
- AlaLeuSerThrThrGlyGluIleProPheTyrGlyLysAlaIleProLeuGluValIle
 TGCTCTGTCCACCACCGGAGAGATCCCTTTTTACGGCAAGGCTATCCCCCTCGAAGTAAT
 ACGAGACAGGTGGTGGCCTCTCTAGGGAAAAATGCCGTTCCGATAGGGGGAGCTTCATTA
- LysLeuValAlaLeuGlyIleAsnAlaValAlaTyrTyrArgGlyLeuAspValSerVal
 AAAGCTGGTCGCATTGGGCATCAATGCCGTGGCCTACTACCGCGGTCTTGACGTGTCCGT
 TTTCGACCAGCGTAACCCGTAGTTACGGCACCGGATGATGGCGCCAGAACTGCACAGGCA

IleProThr
481 CATCCCGACCAG
GTAGGGCTGGTC

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FIG. 13 Translation of DNA 25c

1	CysSerLeuThrValThrGlnLeuLeuArgArgLeuHisGlnTrpIleSerSerGluCys ACTGCAGCCTCACTGTAACCCAGCTCCTGAGGCGACTGCACCAGTGGATAAGCTCGGAGT TGACGTCGGAGTGACATTGGGTCGAGGACTCCGCTGACGTGGTCACCTATTCGAGCCTCA
61	ThrThrProCysSerGlySerTrpLeuArgAspIleTrpAspTrpIleCysGluValLeuGTACCACTCCATGCTCCGGTTCCTGGCTAAGGGACATCTGGGACTGGATATGCGAGGTGTCATGGTGAGGTGAGGTGAGGTGAGGTGAGGTGAGGTGAGGTGAGGTGAGGTGAGGTGAGACCTATACGCTCCACA
121	SerAspPheLysThrTrpLeuLysAlaLysLeuMetProGlnLeuProGlyIleProPheTGAGCGACTTTAAGACCTGACTAAAAGCTAAGCTCATGCCACAGCTGCCTGGGATCCCCTACTCGACTGAAATTCTGGACCGATTTTCGATTCGAGTACGGTGTCGACGGACCCTAGGGGA
181	ValSerCysGlnArgGlyTyrLysGlyValTrpArgGlyAspGlyIleMetHisThrArg TTGTGTCCTGCCAGCGGGGTATAAGGGGGTCTGGCGAGGGGACGGCATCATGCACACTC AACACAGGACGGTCGCGCCCATATTCCCCCAGACCGCTCCCCTGCCGTAGTACGTGTGAG
241	CysHisCysGlyAlaGluIleThrGlyHisValLysAsnGlyThrMetArgIleValGlyGCTGCCACTGTGGAGCTGAGATCACTGGACATGTCAAAAACGGGACGATGAGGATCGTCGCGACGTGACACTCTAGTGACCTGTACAGTTTTTGCCCTGCTACTCCTAGCAGC
301	ProArgThrCysArgAsnMetTrpSerGlyThrPheProIleAsnAlaTyrThrThrGlyGTCCTAGGACCTGCAGGAACATGTGGAGTGGGACCTTCCCCATTAATGCCTACACCACGGCAGGATCCTGGACGTCCTTGTACACCTCACCCTGGAAGGGGTAATTACGGATGTGGTGCC
361	ProCysThrProLeuProAlaProAsnTyrThrPheAlaLeuTrpArgValSerAlaGluGCCCCTGTACCCCCCTTCCTGCGCCGAACTACACGTTCGCGCTATGGAGGGTGTCTGCAGCGGGGGAAGGACGCGGCTTGATGTGCAAGCGCGATACCTCCCACAGACGTC
421	GluTyrValGluIleArgGlnValGlyAspPheHisTyrValThrGlyMetThrThrAsp AGGAATATGTGGAGATAAGGCAGGTGGGGGACTTCCACTACGTGACGGGTATGACTACTG TCCTTATACACCTCTATTCCGTCCACCCCCTGAAGGTGATGCACTGCCCATACTGATGAC
481	AsnLeuLysCysProCysGlnValProSerProGluPhePheThrGlu ACAATCTCAAATGCCCGTGCCAGGTCCCATCGCCCGAATTTTTCACAGAAT TGTTAGAGTTTACGGGCACGGTCCAGGGTAGCGGGCTTAAAAAGTGTCTTA

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- FIG. 14-1 Combined ORF of DNAs 40b/37b/35/36/81/32/33b/25c
 - AlaTyrMetSerLysAlaHisGlyIleAspProAsnIleArgThrGlyValArgThrIle

 TGCTTACATGTCCAAGGCTCATGGGATCGATCCTAACATCAGGACCGGGGTGAGAACAAT
 ACGAATGTACAGGTTCCGAGTACCCTAGCTAGGATTGTAGTCCTGGCCCCACTCTTGTTA
 - ThrThrGlySerProlleThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGlyCys
 61 TACCACTGGCAGCCCCATCACGTACTCCACCTACGGCAAGTTCCTTGCCGACGGCGGTG
 ATGGTGACCGTCGGGGTAGTGCATGAGGTGGATGCCGTTCAAGGAACGGCTGCCGCCCAC
 - SerGlyGlyAlaTyrAspIleIleIleCysAspGluCysHisSerThrAspAlaThrSer
 121 CTCGGGGGGCGCTTATGACATAATAATTTGTGACGAGTGCCACTCCACGGATGCCACATC
 GAGCCCCCCGCGAATACTGTATTATTAAACACTGCTCACGGTGAGGTGCCTACGGTGTAG

 - LeuAlaThrAlaThrProProGlySerValThrValProHisProAsnIleGluGluVal
 GCTCGCCACCGCCACCCCTCCGGGCTCCCTCACTGTGCCCCATCCCAACATCGAGGAGGT
 CGAGCGGTGGCGGTGGGGAGGCCCGAGGCAGTGACACGGGGTAGGGTTGTAGCTCCTCCA
 - AlaLeuSerThrThrGlyGluIleProPheTyrGlyLysAlaIleProLeuGluValIle
 TGCTCTGTCCACCACCGGAGAGATCCCTTTTTACGGCAAGGCTATCCCCCTCGAAGTAAT
 ACGAGACAGGTGGTCGCCTCTCTAGGGAAAAATGCCGTTCCGATAGGGGGAGCTTCATTA

 - LysLeuValAlaLeuGlyIleAsnAlaValAlaTyrTyrArgGlyLeuAspValSerVal
 421 AAAGCTGGTCGCATTGGGCATCAATGCCGTGGCCTACTACCGCGGTCTTGACGTGTCCGT
 TTTCGACCAGCGTAACCCGTAGTTACGGCACCGGATGATGGCGCCAGAACTGCACAGGCA
 - IleProThrSerGlyAspValValValAlaThrAspAlaLeuMetThrGlyTyrThr
 481 CATCCCGACCAGCGGCGATGTTGTCGTCGTGGCAACCGATGCCCTCATGACCGGCTATAC
 GTAGGGCTGGTCGCCGCTACAACAGCAGCACCGTTGGCTACGGGAGTACTGGCCGATATG
 - GlyAspPheAspSerValIleAspTyrAsnThrCysValThrGlnThrValAspPheSer
 CGGCGACTTCGACTCGGTGATAGACTACAATACGTGTGTCACCCAGACAGTCGATTTCAG
 GCCGCTGAAGCTGAGCCACTATCTGATGTTATGCACACAGTGGGTCTGTCAGCTAAAGTC
 - LeuAspProThrPheThrIleGluThrIleThrLeuProGlnAspAlaValSerArgThr
 601 CCTTGACCCTACCTTCACCATTGAGACAATCACGCTCCCCCAGGATGCTGTCTCCCGCAC
 GGAACTGGGATGGAAGTGGTAACTCTGTTAGTGCGAGGGGGTCCTACGACAGAGGGCGTG
 - GlnArgArgGlyArgThrGlyArgGlyLysProGlyIleTyrArgPheValAlaProGly

 661 TCAACGTCGGGGCAGGACTGGCAGGGGAAGCCAGGCATCTACAGATTTGTGGCACCGGGG

 AGTTGCAGCCCCGTCCTGACCGTCCCCTTCGGTCCGTAGATGTCTAAACACCGTGGCCC
 - GluArgProSerGlyMetPheAspSerSerValLeuCysGluCysTyrAspAlaGlyCys
 721 GGAGCGCCCTCCGGCATGTTCGACTCGTCCGTCCTCTGTGAGTGCTATGACGCAGGCTG
 CCTCGCGGGGAGGCCGTACAAGCTGAGCAGGCAGACACTCACGATACTGCGTCCGAC
 - AlaTrpTyrGluLeuThrProAlaGluThrThrValArgLeuArgAlaTyrMetAsnThr
 781 TGCTTGGTATGAGCTCACGCCCGCCGAGACTACAGTTAGGCTACGAGCGTACATGAACAC
 ACGAACCATACTCGAGTGCGGGCGCTCTGATGTCAATCCGATGCTCGCATGTACTTGTG
 - ProGlyLeuProValCysGlnAspHisLeuGluPheTrpGluGlyValPheThrGlyLeu
 841 CCCGGGGCTTCCCGTGTGCCAGGACCATCTTGAATTTTGGGAGGGCGTCTTTACAGGCCT
 GGGCCCCGAAGGGCACACGGTCCTGGTAGAACTTAAAACCCTCCCGCAGAAATGTCCGGA

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961	LeuValAlaTyrGlnAlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAsp CCTGGTAGCGTACCAAGCCACCGTGTGCGCTAGGGCTCAAGCCCCTCCCCCATCGTGGGA GGACCATCGCATGGTTCGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGGTAGCACCCT
1021	GlnMetTrpLysCysLeuIleArgLeuLysProThrLeuHisGlyProThrProLeuLeu CCAGATGTGGAAGTGTTTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGCT GGTCTACACCTTCACAAACTAAGCGGAGTTCGGGTGGGAGGTACCCGGTTGTGGGGACGA
1081	TyrArgLeuGlyAlaValGlnAsnGluIleThrLeuThrHisProValThrLysTyrIle ATACAGACTGGGCGCTGTTCAGAATGAAATCACCCTGACGCACCCAGTCACCAAATACAT TATGTCTGACCCGCGACAAGTCTTACTTTAGTGGGACTGCGTGGGTCAGTGGTTTATGTA
1141	MetThrCysMetSerAlaAspLeuGluValValThrSerThrTrpValLeuValGlyGlyCATGACATGCATGTCGGCCGACCTGGAGGTCGTCACGAGCACCTGGGTGCTCGTTGGCGGGTACTGTACAGCAGCCGGCTGGACCTCCAGCAGTGCTCGTGGACCCACGAGCAACCGCC
1201	ValleuAlaAlaLeuAlaAlaTyrCysLeuSerThrGlyCysValValIleValGlyArg CGTCCTGGCTGCTTTGGCCGCGTATTGCCTGTCAACAGGCTGCGTGGTCATAGTGGGCAG GCAGGACCGACGAAACCGGCGCATAACGGACAGTTGTCCGACGCACCAGTATCACCCGTC
1261	ValValLeuSerGlyLysProAlaIleIleProAspArgGluValLeuTyrArgGluPhe GGTCGTCTTGTCCGGGAAGCCGGCAATCATACCTGACAGGAAGTCCTCTACCGAGAGTT CCAGCAGAACAGGCCCTTCGGCCGTTAGTATGGACTGTCCCTTCAGGAGATGGCTCTCAA
1321	AspGluMetGluGluCysSerGlnHisLeuProTyrIleGluGlnGlyMetMetLeuAla CGATGAGATGGAAGAGTGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCTCGC GCTACTCTACCTTCTCACGAGAGTCGTGAATGGCATGTAGCTCGTTCCCTACTACGAGCG
1381	GluGlnPheLysGlnLysAlaLeuGlyLeuLeuGlnThrAlaSerArgGlnAlaGluVal CGAGCAGTTCAAGCAGAAGGCCCTCGGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGGT GCTCGTCAAGTTCGTCTTCCGGGAGCCGGAGGACGTCTGGCGCAGGGCAGTCCGTCTCCA
1441	IleAlaProAlaValGlnThrAsnTrpGlnLysLeuGluThrPheTrpAlaLysHisMet TATCGCCCCTGCTGTCCAGACCAACTGGCAAAAACTCGAGACCTTCTGGGCGAAGCATAT ATAGCGGGGACGACAGGTCTGGTTGACCGTTTTTGAGCTCTGGAAGACCCGCTTCGTATA
1501	TrpAsnPheIleSerGlyIleGlnTyrLeuAlaGlyLeuSerThrLeuProGlyAsnPro GTGGAACTTCATCAGTGGGATACAATACTTGGCGGGCTTGTCAACGCTGCCTGGTAACCC CACCTTGAAGTAGTCACCCTATGTTATGAACCGCCCGAACAGTTGCGACGGACCATTGGG
1561	AlaIleAlaSerLeuMetAlaPheThrAlaAlaValThrSerProLeuThrThrSerGln CGCCATTGCTTCATTGATGGCTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCCA GCGGTAACGAAGTAACTACCGAAAATGTCGACGACAGTGGTCGGGTGATTGGTGATCGGT
1621	ThrLeuleuPheAsnIleLeuGlyGlyTrpValAlaAlaGlnLeuAlaAlaProGlyAla AACCCTCCTCTTCAACATATTGGGGGGGGTGGGTGGCCCAGCTCGCCGCCCCCGGTGC TTGGGAGGAGAAGTTGTATAACCCCCCCACCCACCGACGGGTCGAGCGGCGGGGGCCACG
1681	AlaThralaPheValGlyAlaGlyLeuAlaGlyAlaAlaIleGlySerValGlyLeuGlyCGCTACTGCCTTTGTGGGCGCTGGCTTAGCTGGCGCCGCCATCGGCAGTGTTGGACTGGGGGCGATGACGGAAACACCCGCGACCGAATCGACCGCGGCGGTAGCCGTCACAACCTGACCC
1741	LysValLeulleAsplleLeuAlaGlyTyrGlyAlaGlyValAlaGlyAlaLeuValAla GAAGGTCCTCATAGACATCCTTGCAGGGTATGGCGCGGGCGTGGCGGAGCTCTTGTGGC CTTCCAGGAGTATCTGTAGGAACGTCCCATACCGCGCCCCGCACCGCCCTCGAGAACACCG
1801	TAAGTTCTAGTACTCGCCACTCCAGGGGAGGTGCCTCCTGGACCAGTTAGATGACGGGCG
1861	GTAGGAGAGCGGCCTCGGGAGCATCAGCCGCACCAGACACGTCGTTATGACGCGGCCGT
FIG. 14	-2 _{ValGlyProGlyGluGlyAlaValGlnTrpMetAsnArgLeuIleAlaPheAlaSerArg}

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1921	CGTTGGCCCGGGCGAGGGGGCAGTGCAGTGGATGAACCGGCTGATAGCCTTCGCCTCCCGGCAACCGGGCCCGCTCCCCCGTCACGTCACCTACTTGGCCGACTATCGGAAGCGGAGGGC
1981	GlyAsnHisValSerProThrHisTyrValProGluSerAspAlaAlaAlaArgValThr GGGGAACCATGTTTCCCCCACGCACTACGTGCCGGAGAGCGATGCAGCTGCCCGCGTCAC CCCCTTGGTACAAAGGGGGTGCGTGATGCACGGCCTCTCGCTACGTCGACGGGCGCAGTG
2041	AlaileLeuSerSerLeuThrValThrGlnLeuLeuArgArgLeuHisGlnTrpIleSer TGCCATACTCAGCAGCCTCACTGTAACCCAGCTCCTGAGGCGACTGCACCAGTGGATAAG ACGGTATGAGTCGTCGGAGTGACATTGGGTCGAGGACTCCGCTGACGTGGTCACCTATTC
2101	SerGluCysThrThrProCysSerGlySerTrpLeuArgAspIleTrpAspTrpIleCys CTCGGAGTGTACCACTCCATGCTCCGGTTCCTGGCTAAGGGACATCTGGGACTGGATATG GAGCCTCACATGGTGAGGTACGAGGCCCAAGGACCGATTCCCTGTAGACCCTGACCTATAC
2161	GluValleuSerAspPheLysThrTrpLeuLysAlaLysLeuMetProGlnLeuProGly CGAGGTGTTGAGCGACTTTAAGACCTGGCTAAAAGCTAAGCTCATGCCACAGCTGCCTGG GCTCCACAACTCGCTGAAATTCTGGACCGATTTTCGATTCGAGTACGGTGTCGACGGACC
2221	IleProPheValSerCysGlnArgGlyTyrLysGlyValTrpArgValAspGlyIleMet GATCCCCTTTGTGTCCTGCCAGCGCGGTATAAGGGGGTCTGGCGAGTGGACGGCATCAT CTAGGGGAAACACAGGACGGTCGCGCCCATATTCCCCCAGACCGCTCACCTGCCGTAGTA
2281	HisThrArgCysHisCysGlyAlaGlulleThrGlyHisValLysAsnGlyThrMetArg GCACACTCGCTGCCACTGTGGAGCTGAGATCACTGGACATGTCAAAAACGGGACGATGAG CGTGTGAGCGACGGTGACACCTCGACTCTAGTGACCTGTACAGTTTTTGCCCTGCTACTC
2341	IleValGlyProArgThrCysArgAsnMetTrpSerGlyThrPheProIleAsnAlaTyr GATCGTCGGTCCTAGGACCTGCAGGAACATGTGGAGTGGGACCTTCCCCATTAATGCCTA CTAGCAGCCAGGATCCTGGACGTCCTTGTACACCTCACCCTGGAAGGGGTAATTACGGAT
2401	ThrThrGlyProCysThrProLeuProAlaProAsnTyrThrPheAlaLeuTrpArgValCACCACGGGCCCCTGTACCCCCCTTCCTGCGCCGAACTACACGTTCGCGCCTATGGAGGGTGTGGTGCCCGGGGACATGGGGGGGAAGGACGCGGCTTGATGTGCAAGCGCGATACCTCCCA
2461	SerAlaGluGluTyrValGlulleArgGlnValGlyAspPheHisTyrValThrGlyMet GTCTGCAGAGGAATATGTGGAGATAAGGCAGGTGGGGGACTTCCACTACGTGACGGGTAT CAGACGTCTCCTTATACACCTCTATTCCGTCCACCCCCTGAAGGTGATGCACTGCCCATA
2521	ThrThrAspAsnLeuLysCysProCysGlnValProSerProGluPhePheThrGluGACTACTGACAATCTCAAATGCCCGTGCCAGGTCCCATCGCCCGAATTTTTCACAGAATCTGATGACTGTTAGAGTTTACGGGCACGGTCCAGGGTAGCGGCCTTAAAAAGTGTCTTA

FIG. 14-3

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FIG. 15 Translation of DNA 33c

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121

- AlavalAspPheIleProValGluAsnLeuGluThrThrMetArgSerProValPheThr
 GGCGGTGGACTTTATCCCTGTGGAGAACCTAGAGACAACCATGAGGTCCCCGGTGTTCAC
 CCGCCACCTGAAATAGGGACACCTCTTGGATCTCTGTTGGTACTCCAGGGGCCACAAGTG

 AspAsnSerSerProProValValProGlnSerPheGlnValAlaHisLeuHisAlaPro
 GGATAACTCCTCTCCACCAGTAGTGCCCCAGAGCTTCCAGGTGGCTCACCTCCATGCTCC
 CCTATTGAGGAGAGGTGGTCATCACGGGGTCTCGAAGGTCCACCGAGTGGAGGTACGAGG

 ThrGlySerGlyLysSerThrLysValProAlaAlaTyrAlaAlaGlnGlyTyrLysVal
 CACAGGCAGCGGCAAAAGCACCAAGGTCCCGGCTGCATATGCAGGTCAGGGCTATAAGGT
 GTGTCCGTCGCCGTTTTCGTGGTTCCAGGGCCGACGTATACGTCGAGTCCCGATATTCCA
- LeuValleuAspProSerValAlaAlaThrLeuGlyPheGlyAlaTyrMetSerLysAla 181 GCTAGTACTCAACCCCTCTGTTGCTGCAACACTGGGCTTTGGTGCTTACATGTCCAAGGC CGATCATGAGTTGGGGAGACAACGACGTTGTGACCCGAAACCACGAATGTACAGGTTCCG
- ThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGlyCysSerGlyGlyAlaTyrAsp
 CACGTACTCCACCTACGGCAAGTTCCTTGCCGACGGCGGGTGCTCGGGGGGGCGCTTATGA
 GTGCATGAGGTGGATGCGGTTCAAGGAACGGCTGCCGCCCACGAGCCCCCCGCGAATACT
- ValleuAspGlnAlaGluThrAlaGlyAlaArgLeuValValLeuAlaThrAlaThrPTO
 421 TGTCCTTGACCAAGCAGAGACTGCGGGGGGGGAGACTGGTTGTGCTCGCCACCGCCACCCC
 ACAGGAACTGGTTCGTCTCTGACGCCCCCGCTCTGACCAACACGAGCGGTGGCGGTGGGGG
- ProGlySerValThrValProHisProAsnIleGluGluValAlaLeuSerThrThrGly
 TCCGGGCTCCGTCACTGTGCCCCATCCCAACATCGAGGAGGTTGCTCTGTCCACCACCACGG
 AGGCCCGAGGCAGTGACACGGGGTAGGGTTGTAGCTCCTCCAACGAGACAGGTGGTGGCC
- IlePheCysHisSerLysLysCysAspGluLeuAlaAlaLysLeuValAlaLeuGly
 601 CATCITCTGTCATTCAAAGAAGAAGTGCGACGAACTCGCCGCAAAGCTGGTCGCATTGGG
 GTAGAAGACAGTAAGTTTCTTCTTCACGCTGCTTGAGCGGCGTTTCGACCAGCGTAACCC
- IleAsnAlaValAlaTyrTyrArgGlyLeuAspValSerValIleProThrSerGlyAsp
 661 CATCAATGCCGTGGCCTACTACCGCGGTCTTGACGTGTCCGTCATCCCGACCAGCGGCGA
 GTAGTTACGGCACCGGATGATGGCGCCAGAACTGCACAGGCAGTAGGGCTGGTCGCCGCT
- ValValValAlaThrAspAlaLeuMetThrGlyTyrThrGlyAspPheAspSerVal
 TGTTGTCGTCGTGGCAACCGATGCCCTCATGACCGGCTATACCGGCGACTTCGACTCGGT
 ACAACAGCAGCACCGTTGGCTACCGGGAGTACTGGCCGATATGGCCGCTGAAGCTGAGCCA
- IleaspcysasnThrCys
 781 GATAGACTGCAATACGTGTG
 CTATCTGACGTTATGCACAC

CURCULITE SHEET

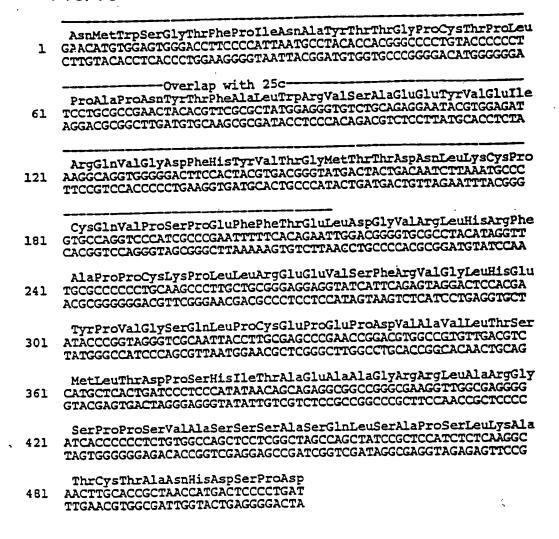
FIG. 16 Translation of DNA 8h ProCysThrCysGlySerSerAspLeuTyrLeuValThrArgHisAlaAspValIlePro CTCCCTGCACTTGCGGCTCCTCGGACCTTTACCTGGTCACGAGGCACGCCGATGTCATTC GAGGGACGTGAACGCCGAGGAGCCTGGAAATGGACCAGTGCTCCGTGCGGCTACAGTAAG ValArgArgArgGlyAspSerArgGlySerLeuLeuSerProArgProIleSerTyrLeu 61 CCGTGCGCCGGCGGGTGATAGCAGGGCAGCCTGCTCTCGCCCCGGCCCATTTCCTACT GGCACGCGGCCCCACTATCGTCCCCGTCGGACGACAGCGGGCCGGGTAAAGGATGA LysGlySerSerGlyGlyProLeuLeuCysProAlaGlyHisAlaValGlyHePheArg 121 TGAAAGGCTCCTCGGGGGGTCCGCTGTTGTGCCCCGCGGGGCACGCCGTGGGCATATTTA ACTTTCCGAGGAGCCCCCCAGGCGACAACACGGGGCGCCCCGTGCGGCACCCGTATAAAT -Overlap with AlaAlaValCysThrArgGlyValAlaLysAlaValAspPheIleProValGluAsnLeu 181 GGGCCGCGTGTGCACCCGTGGAGTGGCTAAGGCGGTGGACTTTATCCCTGTGGAGAACC CCCGGCGCCACACGTGGGCACCTCACCGATTCCGCCACCTGAAATAGGGACACCTCTTGG GluThrThrMetArgSerProValPheThrAspAsnSer TAGAGACAACCATGAGGTCCCCGGTGTTCACGGATAACTCCTC ATCTCTGTTGGTACTCCAGGGGCCACAAGTGCCTATTGAGGAG FIG. 17 Translation of DNA 7e GlyTrpArgLeuLeuAlaProIleThrAlaTyrAlaGlnGlnThrArgGlyLeuLeuGly GGGGTGGAGGTTGCTGGCGCCCATCACGGCGTACGCCCAGCAGACAAGGGGCCTCCTAGG CCCCACCTCCAACGACCGCGGGTAGTGCCGCATGCGGGTCGTCTGTTCCCCGGAGGATCC CysIleIleThrSerLeuThrGlyArgAspLysAsnGlnValGluGlyGluValGlnIle GTGCATAATCACCAGCCTAACTGGCCGGGACAAAAACCAAGTGGAGGGTGAGGTCCAGAT CACGTATTAGTGGTCGGATTGACCGGCCCTGTTTTTGGTTCACCTCCCACTCCAGGTCTA ValSerThrAlaAlaGlnThrPheLeuAlaThrCysIleAsnGlyValCysTrpThrVal TGTGTCAACTGCTGCCCAAACCTTCCTGGCAACGTGCATCAATGGGGTGTGCTGGACTGT ACACAGTTGACGACGGGTTTGGAAGGACCGTTGCACGTAGTTACCCCACACGACCTGACA TyrHisGlyAlaGlyThrArgThrIleAlaSerProLysGlyProValIleGlnMetTyr CTÁCCACGGGGCCGGÁACGAGGACCATCGCGTCACCCAÁGGGTCCTGTCATCCAGATGTA GATGGTGCCCCGGCCTTGCTCCTGGTAGCGCAGTGGGTTCCCAGGACAGTAGGTCTACAT ThrAsnValAspGlnAspLeuValGlyTrpProAlaProGlnGlySerArgSerLeuThr TACCAATGTAGACCAAGACCTTGTGGGCTGGCCGGCTCGCAAGGTAGCCGCTCATTGAC 241 ATGGTTACATCTGGTTCTGGAACACCCGACCGGGCGAGGCGTTCCATCGGCGAGTAACTG

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ProCysThrCysGlySerSerAspLeuTyrLeuValThrArgHis
301 ACCCTGCACTTGCGGCTCCTCGGACCTTTACCTGGTCACGAGGCACG
TGGGACGTGAACGCCGAGGAGCCTGGAAATGGACCAGTGCTCCGTGC

-Overlap with 8h-

FIG. 18 Translation of DNA 14c



SUBSTITUTE SHEET

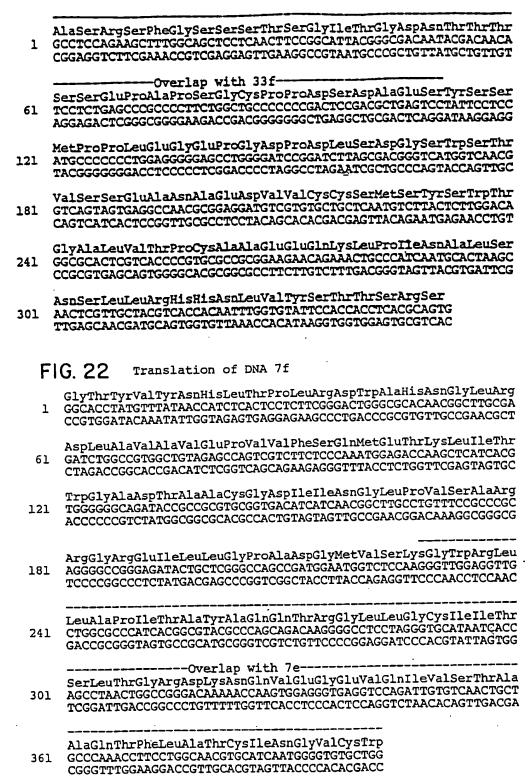
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FIG. 19 Translation of DNA 8f -Overlap with 14c-SerSerSerAlaSerGlnLeuSerAlaProSerLeuLysAlaThrCysThrAlaAsnHis AGCTCCTCGGCTAGCCAGCTATCCGCTCCATCTCTCAAGGCAACTTGCACCGCTAACCAT TCGAGGAGCCGATCGGTCGATAGGCGAGGTAGAGAGTTCCGTTGAACGTGGCGATTGGTA AspSerProAspAlaGluLeuIleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGly 61 GACTCCCTGATGCTGAGCTCATAGAGGCCAACCTCCTATGGAGGCAGGAGATGGGCGGC CTGAGGGGACTACGACTCGAGTATCTCCGGTTGGAGGATACCTCCGTCCTCTACCCGCCG AsnIleThrArgValGluSerGluAsnLysValValIleLeuAspSerPheAspProLeu 121 AACATCACCAGGGTTGAGTCAGAAAACAAAGTGGTGATTCTGGACTCCTTCGATCCGCTT TTG TAGTGGTCCCAACTCAGTCTTTTGTTTCACCACTAAGACCTGAGGAAGCTAGGCGAA ValAlaGluGluAspGluArgGluIleSerValProAlaGluIleLeuArgLysSerArg GTGGCGGAGGACGACCGGGAGATCTCCGTACCCGCAGAAATCCTGCGGAAGTCTCGG CACCGCCTCCTCCTCCCCCCCTCTAGAGGCATGGGCGTCTTTAGGACGCCTTCAGAGCC ArgPheAlaGlnAlaLeuProValTrpAlaArgProAspTyrAsnProProLeuValGlu AGATTCGCCCAGGCCCGCTTTGGGCGCGGCCGGACTATAACCCCCCGCTAGTGGAG TCTAAGCGGGTCCGGGACGGGCAAACCCGCGCCGGCCTGATATTGGGGGGGCGATCACCTC ThrTrpLysLysProAspTyrGluProProValValHisGlyCysProLeuProProPro 301 ACGTGGAAAAAGCCCGACTACGAACCACCTGTGGTCCATGGCTGTCCGCTTCCACCTCCA TGCACCTTTTTCGGGCTGATGCTTGGTGGACACCAGGTACCGACAGGCGAAGGTGGAGGT LysSerProProValPro 361 AAGTCCCCTCCTGTGCCG TTCAGGGGAGGACACGGC FIG. 20 Translation of DNA 33f ValTrpAlaArgProAspTyrAsnProProLeuValGluThrTrpLysLysProAspTyr CGTTTGGGCGCGGCCGGACTĂTAACCCCCCGCTAGTGGAGACGTGGAĀAAĀACCCGACTĀ GCAAACCCGCGCCGGCCTGATATTGGGGGGGGGATCACCTCTGCACCTTTTTTGGGCTGAT -Overlap with 8f-GluProProValValHisGlyCysProLeuProProProLysSerProProValProPro CGAACCACCTGTGGTCCATGGCTGCCCGCTTCCACCTCCAAAGTCCCCTCCTGTGCCTCC GCTTGGTGGACACCAGGTACCGACGGGCGAAGGTGGAGGTTTCAGGGGAGGACACGGAGG ProArglysLysArgThrValValleuThrGluSerThrLeuSerThrAlaLeuAlaGlu 121 GCCTCGGAAGAAGCGGACGGTGGTCCTCACTGAATCAACCCTATCTACTGCCTTGGCCGA CGGAGCCTTCTTCGCCTGCCACCAGGAGTGACTTAGTTGGGATAGATGACGGAACCGGCT LeuAlaThrArgSerPheGlySerSerSerThrSerGlyIleThrGlyAspAsnThrThr 181 GCTCGCCACCAGAAGCTTTGGCAGCTCCTCAACTTCCGGCATTACGGGCGACAATACGAC CGAGCGGTGGTCTTCGAAACCGTCGAGGAGTTGAAGGCCGTAATGCCCGCTGTTATGCTG ThrSerSerGluProAlaProSerGlyCysProProAspSerAspAlaGluSerPhe

SURSTITUTE SHEET

AACATCCTCTGAGCCCGCCCCTTCTGGCTGCCCCCGACTCCGACGCTGAGTCCTTTGC TTGTAGGAGACTCGGGCGGGAAGACCGACGGGGGGGGCTGAGGCTGCGACTCAGGAAACG

FIG. 21 Translation of DNA 33g



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FIG. 23 Translation of DNA 11b

- GlyGlyValValLeuValGlyLeuMetAlaLeuThrLeuSerProTyrTyrLysArgTyr 1 GGCGGTGTTGTTCTCGTCGGGTTGATGGCGCTGACTCTGTCACCATATTACAAGCGCTAT CCGCCACAACAAGAGCAGCCCAACTACCGCGACTGAGACAGTGGTATAATGTTCGCGATA
- IleSerTrpCysLeuTrpTrpLeuGlnTyrPheLeuThrArgValGluAlaGlnLeuHis 61 ATCAGCTGGTGCTTGTGGTGGCTTCAGTATTTTCTGACCAGAGTGGAAGCGCAACTGCAC TAGTCGACCACGAACACCACCGAAGTCATAAAAGACTGGTCTCACCTTCGCGTTGACGTG
- ValTrpIleProProLeuAsnValArgGlyGlyArgAspAlaValIleLeuLeuMetCys GTGTGGATTCCCCCCTCAACGTCCGAGGGGGGCGCGACGCCGTCATCTTACTCATGTGT CACACCTAAGGGGGGGAGTTGCAGGCTCCCCCCGCGCTGCGGCAGTAGAATGAGTACACA
- AlaValHisProThrLeuValPheAspIleThrLysLeuLeuAlaValPheGlyPro GCTGTACACCCGACTCTGGTATTTGACATCACCAAATTGCTGGCCGTCTTCGGACCC CGACATGTGGGCTGAGACCATAAACTGTAGTGGTTTAACGACGACCGGCAGAAGCCTGGG
- LeuTrpIleLeuGlnAlaSerLeuLeuLysValProTyrPheValArgValGlnGlyLeu
 241 CTTTGGATTCTTCAAGCCAGTTTGCTTAAAGTACCCTACTTTGTGCGCGTCCAAGGCCTT
 GAAACCTAAGAAGTTCGGTCAAACGAATTTCATGGGATGAAACACGCGCAGGTTCCGGAA
- LeuArgPheCysAlaLeuAlaArgLysMetlleGlyGlyHisTyrValGlnMetVallle
 301 CTCCGGTTCTGCGCGTTAGCGCGGAAGATGATCGGAGGCCATTACGTGCAAATGGTCATC
 GAGGCCAAGACGCGCAATCGCGCCTTCTACTAGCCTCCGGTAATGCACGTTTACCAGTAG
- IleLysLeuGlyAlaLeuThrGlyThrTyrValTyrAsnHisLeuThrProLeuArgAsp 361 ATTAAGTTAGGGGCGCTTACTGGCACCTATGTTTATAACCATCTCACTCCTCTTCGGGAC TAATTCAATCCCCGCGAATGACCGTGGATACAAATATTGGTAGAGTGAGGAGAAGCCCTG
- TrpAlaHisAsnGlyLeuArgAspLeuAlaValAlaValGluProValValPheSerGln
 TGGGCGCACAACGGCTTGCGAGATCTGGCCGTGGCTGTAGAGCCAGTCGTCTCTCCCAA
 ACCCGCGTGTTGCCGAACGCTCTAGACCGGCACCGACATCTCGGTCAGCAGAAGAGGGTT
- MetGluThrLysLeuIleThrTrpGly
 481 ATGGAGACCAAGCTCATCACGTGGGGGGC
 TACCTCTGGTTCGAGTAGTGCACCCCCG

CHRETITIF CHEET

_	21C 24
	IG. 24 Translation of DNA 14i
1	GluTyrValValLeuLeuPheLeuLeuLeuAlaAspAlaArgValCysSerCysLeuTrp GGGAGTACGTCGTTCTCTGTTCCTTGCTTGCAGACGCGCGCG
61	MetMetLeuLeuIleSerGlnAlaGluAlaAlaLeuGluAsnLeuValIleLeuAsnAla GGATGATGCTACTCATATCCCAAGCGGAGGCGGCTTTGGAGAACCTCGTAATACTTAATG CCTACTACGATGAGTATAGGGTTCGCCTCCGCCGAAACCTCTTGGAGCATTATGAATTAC
121	AlaSerLeuAlaGlyThrHisGlyLeuValSerPheLeuValPhePheCysPheAlaTrp CAGCATCCCTGGCCGGGACGCACGGTCTTGTATCCTTCCT
181	TyrLeuLysGlyLysTrpValProGlyAlaValTyrThrPheTyrGlyMetTrpProLeu GGTATTTGAAGGGTAAGTGGGTGCCCGGAGCGGTCTACACCTTCTACGGGATGTGGCCTC CCATAAACTTCCCATTCACCCACGGGCCTCGCCAGATGTGGAAGATGCCCTACACCGGAG
241	LeuLeuLeuLeuAlaLeuProGlnArgAlaTyrAlaLeuAspThrGluValAlaAla TCCTCCTGCTCCTGTTGGCGTTGCCCCAGCGGGCGTACGCGCCTGGACACGGAGGTGGCCG AGGAGGACGAGGACAACCGCAACGGGGTCGCCCGCATGCGCGACCTGTGCCTCCACCGGC
301	SerCysGlyGlyValValLeuValGlyLeuMetAlaLeuThrLeuSerProTyrTyrLys CGTCGTGTGGCGGTGTTGTTCTCGTCGGGTTGATGGCGCTGACTCTGTCACCATATTACA GCAGCACACCGCCACAACAAGAGCAGCCCCAACTACCGCGACTGAGACAGTGGTATAATGT
361	ArgTyrIleSerTrpCysLeuTrpTrpLeuGln AGCGCTATATCAGCTGGTGCTTGTGGTGGCTTCAGAA TCGCGATATAGTCGACCACCGAACACCACCGAAGTCTT
F	G. 25 Translation of DNA 39c
•	
1	ProAlaProSerGlyCysProProAspSerAspAlaGluSerTyrSerSerMetProPro CCAGCCCCTTCTGGCTGCCCCCCGACTCCGACGCTGAGTCCTATTCCTCCATGCCCCCC GGTCGGGGAAGACCGACGGGGGGCTGAGGCTGCGACTCAGGATAAGGAGGTACGGGGGG
61	LeuGluGlyGluProGlyAspProAspLeuSerAspGlySerTrpSerThrValSerSer CTGGAGGGGGAGCCTGGGGATCCGGATCTTAGCGACGGGTCATGGTCAACAGTCAGT
	Overlap with 33g
121	GlualaasnalagluaspvalvalCysCysSerMetSerTyrSerTrpThrglyAlaLeu GAGGCCAACGCGGAGGATGTCGTGTGCTGCTCAATGTCCTACTCTTGGACAGGCGCACTC CTCCGGTTGCGCCTCCTACAGCACGACGAGTTACAGGATGAGAACCTGTCCGCGTGAG
181	ValThrProCysAlaAlaGluGluGlnLysLeuProIleAsnAlaLeuSerAsnSerLeu GTCACCCCGTGCGCGCGGAAGAACAGAAACTGCCCATCAATGCACTGAGCAACTCGTTG CAGTGGGGCACGCGGCGCCTTCTTGTCTTTGACGGGTAGTTACGTGACTCGTTGAGCAAC
241	LeuArgHisHisAsnLeuValTyrSerThrThrSerArgSerAlaCysGlnArgGlnLys CTACGTCACCACATTTGGTGTATTCCACCACCTCACGCAGTGCTTGCCAAAGGCAGAAG GATGCAGTGGTGTTAAACCACATAAGGTGGTGGAGTGCGTCACGAACGGTTTCCGTCTTC
301	LysValThrPheAspArgLeuGlnValLeuAspSerHisTyrGlnAspValLeuLysGlu AAAGTCACATTTGACAGACTGCAAGTTCTGGACAGCCATTACCAGGACGTACTCAAGGAG TTTCAGTGTAAACTGTCTGACGTTCAAGACCTGTCGGTAATGGTCCTGCATGAGTTCCTC
361	VallysAlaAlaAlaSerLysVallysAlaAsnPhe GTTAAAGCAGCGGCGTCAAAAGTGAAGGCTAACTTC

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- FIG. 26-I COMBINED ORF OF DNAs 141/11b/7f/7e/8h/33c/40b/37b/35/36/81/32/33b/25c/14c/8f/33f/33g/39c
- MetMetLeuLeuIleSerGlnAlaGluAlaAlaLeuGluAsnLeuValIleLeuAsnAla
 61 GGATGATGCTACTCATATCCCAAGCGGAGGCGGCTTTGGAGAACCTCGTAATACTTAATG
 CCTACTACGATGAGTATAGGGTTCGCCTCCGCCGAAACCTCTTGGAGCATTATGAATTAC
- TyrLeuLysGlyLysTrpValProGlyAlaValTyrThrPheTyrGlyMetTrpProLeu
 181 GGTATTTGAAGGGTAAGTGGGTGCCCGGAGCGGTCTACACCTTCTACGGGATGTGGCCTC
 CCATAAACTTCCCATTCACCCACGGGCCTCGCCAGATGTGGAAGATGCCCTACACCGGAG
- LeuLeuLeuLeuAlaLeuProGlnArgAlaTyrAlaLeuAspThrGluValAlaAla
 TCCTCCTGCTCCTGTTGGCGTTGCCCCAGCGGGCGTACGCGCTGGACACGGAGGTGGCCG
 AGGAGGACGAGGACAACCGCAACGGGGTCGCCCGCATGCGCGACCTGTGCCTCCACCGGC
- SerCysGlyGlyValValLeuValGlyLeuMetAlaLeuThrLeuSerProTyrTyrLys
 CGTCGTGTGGCGGTGTTGTTCTCGTCGGGTTGATGGCGCTGACTCTGTCACCATATTACA
 GCAGCACACCGCCACAACAAGAGCAGCCCAACTACCGCGACTGAGACAGTGGTATAATGT
- ArgtyrileSerTrpCysLeuTrpTrpLeuGlnTyrPheLeuThrArgValGluAlaGln AGCGCTATATCAGCTGGTGCTTGTGGTGGCTTCAGTATTTTCTGACCAGAGTGGAAGCGC TCGCGATATAGTCGACCACGAACACCACCGAAGTCATAAAAGACTGGTCTCACCTTCGCG
- MetCysAlaValHisProThrLeuValPheAsplleThrLysLeuLeuAlaValPhe
 481 TCATGTGTGTGTACACCCGACTCTGGTATTTGACATCACCAAATTGCTGCTGGCCGTCT
 AGTACACACGACATGTGGGCTGAGACCATAAACTGTAGTGGTTTAACGACGACCGGCAGA
- GlyProLeuTrpIleLeuGlnAlaSerLeuLeuLysValProTyrPheValArgValGln
 TCGGACCCCTTTGGATTCTTCAAGCCAGTTTGCTTAAAGTACCCTACTTTGTGCGCGTCC
 AGCCTGGGGAAACCTAAGAAGTTCGGTCAAACGAATTTCATGGGATGAAACACGCGCAGG
- GlyLeuLeuArgPheCysAlaLeuAlaArgLysMetIleGlyGlyHisTyrValGlnMet
 AAGGCCTTCTCCGGTTCTGCGCGTTAGCGCGGAAGATGATCGGAGGCCATTACGTGCAAA
 TTCCGGAAGAGGCCAAGACGCGCAATCGCGCCTTCTACTAGCCTCCGGTAATGCACGTTT
- ValileIleLysLeuGlyAlaLeuThrGlyThrTyrValTyrAsnHisLeuThrProLeu
 661 TGGTCATCATTAAGTTAGGGGCGCTTACTGGCACCTATGTTTATAACCATCTCACTCCTC
 ACCAGTAGTAATTCAATCCCCGCGAATGACCGTGGATACAAATATTGGTAGAGTGAGGAG
- ArgAspTrpAlaHisAsnGlyLeuArgAspLeuAlaValAlaValGluProValValPhe
 721 TTCGGGACTGGGCGCACAACGGCTTGCGAGATCTGGCCGTGGCTGTAGAGCCAGTCGTCT
 AAGCCCTGACCCGCGTGTTGCCGAACGCTCTAGACCGGCACCGACATCTCGGTCAGCAGA
- SerGlnMetGluThrLysLeuIleThrTrpGlyAlaAspThrAlaAlaCysGlyAspIle
 781 TCTCCCAAATGGAGACCAAGCTCATCACGTGGGGGGCAGATACCGCCGCGTGACA
 AGAGGGTTTACCTCTGGTTCGAGTAGTGCACCCCCCGTCTATGGCGGCGCACGCCACTGT
- IleAsnGlyLeuProValSerAlaArgArgGlyArgGluIleLeuLeuGlyProAlaAsp
 TCATCAACGGCTTGCCTGTTTCCGCCCGCAGGGGCCCGGAGATACTGCTCGGGCCAGCCG
 AGTAGTTGCCGAACGGACAAAGGCGGGCGTCCCCGGCCCTCTATGACGAGCCCGGTCGGC

 ${\tt GlyMetValSerLysGlyTrpArgLeuLeuAlaProIleThrAlaTyrAlaGlnGlnThr}$

CURCULTUTE SHEET

901	ATGGAATGGTCTCCAAGGGGTGGAGGTTGCTGGCGCCCATCACGGCGTACGCCCAGCAGA TACCTTACCAGAGGTTCCCCACCTCCAACGACGGCGGGTAGTGCCGCATGCGGGTCGTCT
961	ArgGlyLeuLeuGlyCysIleIleThrSerLeuThrGlyArgAspLysAsnGlnValGluCAAGGGGCCTCCTAGGGTGCATAATCACCAGCCTAACTGGCCGGGACAAAAACCAAGTGGGTCCCCGGAGGATCCCACGTATTAGTGGTCGGATTGACCGGCCCTGTTTTTGGTTCACC
1021	GlyGluValGlnIleValSerThrAlaAlaGlnThrPheLeuAlaThrCysIleAsnGly AGGGTGAGGTCCAGATTGTGTCAACTGCTGCCCAAACCTTCCTGGCAACGTGCATCAATG TCCCACTCCAGGTCTAACACAGTTGACGACGGGTTTGGAAGGACCGTTGCACGTAGTTAC
1081	ValCysTrpThrValTyrHisGlyAlaGlyThrArgThrIleAlaSerProLysGlyPro GGGTGTGCTGGACTGTCTACCACGGGGCCCGGACGAGGACCATCGCGTCACCCAAGGGTC CCCACACGACCTGACAGATGGTGCCCCCGGCCTTGCTCCTGGTAGCGCAGTGGGTTCCCAG
1141	ValileGlnMetTyrThrAsnValAspGlnAspLeuValGlyTrpProAlaProGlnGlyCTGTCATCCAGATGTATACCAATGTAGACCAAGACCTTGTGGGCTGGCCCGCTCCGCAAGGACAGTAGGTCTACATATGGTTACATCTGGTTCTGGAACACCCGACCGGGCGAGGCGTTC
1201	SerArgSerLeuThrProCysThrCysGlySerSerAspLeuTyrLeuValThrArgHis GTAGCCGCTCATTGACACCCTGCACTTGCGGCTCCTCGGACCTTTACCTGGTCACGAGGC CATCGGCGAGTAACTGTGGGACGTGAACGCCGAGGAGCCTGGAAATGGACCAGTGCTCCG
1261	AlaAspValileProValArgArgArgGlyAspSerArgGlySerLeuLeuSerProArg ACGCCGATGTCATTCCCGTGCGCCGGCGGGGGTGATAGCAGGGGCAGCCTGCTGTCGCCCC TGCGGCTACAGTAAGGGCACGCGGCCGCCCCACTATCGTCCCCGTCGGACGACAGCGGGG
1321	ProlleSerTyrLeuLysGlySerSerGlyGlyProLeuLeuCysProAlaGlyHisAla GGCCCATTTCCTACTTGAAAGGCTCCTCGGGGGGTCCGCTGTTGTGCCCCGCGGGGCACG CCGGGTAAAGGATGAACTTTCCGAGGAGCCCCCCAGGCGACAACACGGGGCGCCCCGTGC
1381	ValGlyIlePheArgAlaAlaValCysThrArgGlyValAlaLysAlaValAspPheIle CCGTGGGCATATTTAGGGCCGCGGTGTGCACCCGTGGAGTGGCTAAGGCGGTGGACTTTA GGCACCCGTATAAATCCCGGCGCCACACGTGGGCACCTCACCGATTCCGCCACCTGAAAT
1441	ProvalgluasnleugluthrthrmetargserProvalPhethraspasnserSerPro TCCCTGTGGAGAACCTAGAGACAACCATGAGGTCCCCGGTGTTCACGGATAACTCCTCTC AGGGACACCTCTTGGATCTCTGTTGGTACTCCAGGGGCCACAAGTGCCTATTGAGGAGAG
1501	ProvalValProGlnSerPheGlnValAlaHisLeuHisAlaProThrGlySerGlyLys CACCAGTAGTGCCCCAGAGCTTCCAGGTGGCTCACCTCCATGCTCCCACAGGCAGCGGCA GTGGTCATCACGGGGTCTCGAAGGTCCACCGAGTGGAGGTACGAGGGTGTCCGTCGCCGT
1561	SerThrLysValProAlaAlaTyrAlaAlaGlnGlyTyrLysValLeuValLeuAsnPro AAAGCACCAAGGTCCCGGCTGCATATGCAGCTCAGGGCTATAAGGTGCTAGTACTCAACC TTTCGTGGTTCCAGGGCCGACGTATACGTCGAGTCCCGATATTCCACGATCATGAGTTGG
1621	SerValAlaAlaThrLeuGlyPheGlyAlaTyrMetSerLysAlaHisGlyIleAspPro CCTCTGTTGCTGCAACACTGGGCTTTGGTGCTTACATGTCCAAGGCTCATGGGATCGATC
1681	AsnileArgThrGlyValArgThrIleThrThrGlySerProlleThrTyrSerThrTyr CTAACATCAGGACCGGGTGAGAACAATTACCACTGGCAGCCCCATCACGTACTCCACCT GATTGTAGTCCTGGCCCCACTCTTGTTAATGGTGACCGTCGGGGTAGTGCATGAGGTGGA
1741	GlyLysPheLeuAlaAspGlyGlyCysSerGlyGlyAlaTyrAspIleIleIleCysAsp ACGGCAAGTTCCTTGCCGACGGCGGGTGCTCGGGGGGCGCTTATGACATAATATTTGTG TGCCGTTCAAGGAACGGCTGCCGCCCACGAGCCCCCCGCGAATACTGTATTATAAACAC
1801	GluCysHisSerThrAspAlaThrSerIleLeuGlyIleGlyThrValLeuAspGlnAla ACGAGTGCCACTCCACGGATGCCACATCCATCTTGGGCATCGGCACTGTCCTTGACCAAG TGCTCACGGTGAGGTGCCTACGGTGTAGGTAGAACCCGTAGCCGTGACAGGAACTGGTTC
1861	GluThrAlaGlyAlaArgLeuValValLeuAlaThrAlaThrProProGlySerValThr CAGAGACTGCGGGGGCGAGACTGGTTGTGCTCGCCACCCGCCACCCCTCCGGGCTCCGTCA GTCTCTGACGCCCCCGCTCTGACCAACACGAGCGGTGGCGGTGGGGAGGCCCGAGGCAGT
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1921	ValProHisProAsnIleGluGluValAlaLeuSerThrThrGlyGluIleProPheTyr CTGTGCCCCATCCCAACATCGAGGAGGTTGCTCTGTCCACCACCGGAGAGATCCCTTTTT GACACGGGGTAGGGTTGTAGCTCCTCCAACGAGACAGGTGGTGGCCTCTCTAGGGAAAAA
1981	GlyLysAlaIleProLeuGluValIleLysGlyGlyArgHisLeuIlePheCysHisSer ACGGCAAGGCTATCCCCCTCGAAGTAATCAAGGGGGGGAGACATCTCATCTTCTGTCATT TGCCGTTCCGATAGGGGGAGCTTCATTAGTTCCCCCCCTCTGTAGAGTAGAAGACAGTAA
2041	LysLysLysCysAspGluLeuAlaAlaLysLeuValAlaLeuGlyIleAsnAlaValAla CAAAGAAGAAGTGCGACGACTCGCCGCAAAGCTGGTCGCATTGGGCATCAATGCCGTGG GTTTCTTCACGCTGCTTGAGCGGCGTTTCGACCAGCGTAACCCGTAGTTACGGCACC
2101	TyrTyrArgGlyLeuAspValSerValIleProThrSerGlyAspValValValValAla CCTACTACCGCGGTCTTGACGTGTCCGTCATCCCGACCAGCGGCGATGTTGTCGTCGTGG GGATGATGGCGCCAGAACTGCACAGGCAGTAGGGCTGGTCGCCGCTACAACAGCAGCACC
2161	ThrAspAlaLeuMetThrGlyTyrThrGlyAspPheAspSerVallleAspCysAsnThr CAACCGATGCCCTCATGACCGGCTATACCGGCGACTTCGACTCGGTGATAGACTGCAATA GTTGGCTACGGGAGTACTGGCCGATATGGCCGCTGAAGCTGAGCCACTATCTGACGTTAT
2221	CysValThrGlnThrValAspPheSerLeuAspProThrPheThrIleGluThrIleThr CGTGTGTCACCCAGACAGTCGATTTCAGCCTTGACCCTACCTTCACCATTGAGACAATCA GCACACAGTGGGTCTGTCAGCTAAAGTCGGAACTGGGAAGTGGTAACTCTGTTAGT
2281	LeuProGlnAspAlaValSerArgThrGlnArgArgGlyArgThrGlyArgGlyLysPro CGCTCCCCCAGGATGCTGTCTCCCGCACTCAACGTCGGGGCAGGACTGGCAGGGGGAAGC GCGAGGGGGTCCTACGACAGAGGGCGTGAGTTGCAGCCCCGTCCTGACCGTCCCCTTCG
2341	GlylleTyrArgPheValAlaProGlyGluArgProSerGlyMetPheAspSerSerVal CAGGCATCTACAGATTTGTGGCACCGGGGGAGCGCCCCTCCGGCATGTTCGACTCGTCCG GTCCGTAGATGTCTAAACACCGTGGCCCCCTCGCGGGGAGGCCGTACAAGCTGAGCAGGC
2401	LeuCysGluCysTyrAspAlaGlyCysAlaTrpTyrGluLeuThrProAlaGluThrThr TCCTCTGTGAGTGCTATGACGCAGGCTGTGCTTGGTATGAGCTCACGCCCGCC
2461	ValArgLeuArgAlaTyrMetAsnThrProGlyLeuProValCysGlnAspHisLeuGlu CAGTTAGGCTACGAGCGTACATGAACACCCCGGGGCTTCCCGTGTGCCAGGACCATCTTG GTCAATCCGATGCTCGCATGTACTTGTGGGGCCCCGAAGGGCACACGGTCCTGGTAGAAC
2521	PheTrpGluGlyValPheThrGlyLeuThrHisIleAspAlaHisPheLeuSerGlnThr AATTTTGGGAGGGCGTCTTTACAGGCCTCACTCATATAGATGCCCACTTTCTATCCCAGA TTAAAACCCTCCCGCAGAAATGTCCGGAGTGAGTATATCTACGGGTGAAAGATAGGGTCT
2581	LysGlnSerGlyGluAsnLeuProTyrLeuValAlaTyrGlnAlaThrValCysAlaArg CAAAGCAGAGTGGGGAGAACCTTCCTTACCTGGTAGCGTACCAAGCCACCGTGTGCGCTA GTTTCGTCTCACCCCTCTTGGAAGGAATGGACCATCGCATGGTTCGGTGGCACACGCGAT
2641	AlaGlnAlaProProProSerTrpAspGlnMetTrpLysCysLeuIleArgLeuLysProGGGCTCAAGCCCCTCCCCCATCGTGGGACCAGATGTGGAAGTGTTTGATTCGCCTCAAGCCCCCGAGTTCGGGGAGGGGGGAGCACCCTGGTCTACACCTTCACAAACTAAGCGGAGTTCG
2701	ThrLeuHisGlyProThrProLeuLeuTyrArgLeuGlyAlaValGlnAsnGluIleThr CCACCCTCCATGGGCCAACACCCCTGCTATACAGACTGGGCGCTGTTCAGAATGAAATCA GGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGACCCGCGACAAGTCTTACTTTAGT
2761	LeuThrHisProValThrLysTyrIleMetThrCysMetSerAlaAspLeuGluValValCCCTGACGCACCCAGTCACCAAATACATCATGACATGCATG
2821	ThrSerThrTrpValleuValGlyGlyValleuAlaAlaLeuAlaAlaTyrCysLeuSexTCACGAGCACCTGGGTGCTCGTTGGCGGCGTCCTGGCTGCTTTGGCCGCGTATTGCCTGTAGGGGGCCCGCACGAGCAACCGGCGCACGAGCAACCGGCGCACGAGCAACCGGCGACGA
	ThrGlyCysValValIleValGlyArgValValLeuSerGlyLysProAlaIleIlePro
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	25/63
2881	CAACAGGCTGCGTGGTCATAGTGGGCAGGGTCGTCTTGTCCGGGAAGCCGGCAATCATAC GTTGTCCGACGCACCAGTATCACCCGTCCCAGCAGAACAGGCCCTTCGGCCGTTAGTATG
2941	AspArgGluValLeuTyrArgGluPheAspGluMetGluGluCysSerGlnHisLeuPro CTGACAGGGAAGTCCTCTACCGAGAGTTCGATGAGATGGAAGAGTGCTCTCAGCACTTAC GACTGTCCCTTCAGGAGATGGCTCTCAAGCTACTCTACCTTCTCACGAGAGTCGTGAATG
3001	TyrlleGluGlnGlyMetMetLeuAlaGluGlnPheLysGlnLysAlaLeuGlyLeuLeu CGTACATCGAGCAAGGGATGATGCTCGCCGAGCAGTTCAAGCAGAAGGCCCTCGGCCTCC GCATGTAGCTCGTTCCCTACTACGAGCGGCTCGTCAAGTTCGTCTTCCGGGAGCCGGAGG
3061	GlnThrAlaSerArgGlnAlaGluValIleAlaProAlaValGlnThrAsnTrpGlnLys TGCAGACCGCGTCCCGTCAGGCAGAGGTTATCGCCCCTGCTGTCCAGACCAACTGGCAAA ACGTCTGGCGCAGGGCAGTCCGTCTCCAATAGCGGGGACGACAGGTCTGGTTGACCGTTT
3121	LeuGluThrPheTrpAlaLysHisMetTrpAsnPhelleSerGlyIleGlnTyrLeuAla AACTCGAGACCTTCTGGGCGAAGCATATGTGGAACTTCATCAGTGGGATACAATACTTGG TTGAGCTCTGGAAGACCCGCTTCGTATACACCTTGAAGTAGTCACCCTATGTTATGAACC
3181	GlyLeuSerThrLeuProGlyAsnProAlaIleAlaSerLeuMetAlaPheThrAlaAla CGGGCTTGTCAACGCTGCCTGGTAACCCCGCCATTGCTTCATTGATGGCTTTTACAGCTG GCCCGAACAGTTGCGACGGACCATTGGGGCGGTAACGAAGTAACTACCGAAAATGTCGAC
3241	ValThrSerProleuThrThrSerGlnThrLeuLeuPheAsnIleLeuGlyGlyTrpVal CTGTCACCAGCCCACTAACCACTAGCCAAACCCTCCTCTTCAACATATTGGGGGGGTGGG GACAGTGGTCGGGTGATTGGTGATCGGTTTGGGAGGAGAAGTTGTATAACCCCCCCACCC
3301	AlaAlaGlnLeuAlaAlaProGlyAlaAlaThrAlaPheValGlyAlaGlyLeuAlaGly TGGCTGCCCAGCTCGCCCCCCGGTGCCGCTACTGCCTTTGTGGGCGCTGGCTTAGCTG ACCGACGGGTCGAGCGGCGGCGCCACGGCGATGACGGAAACACCCGGGACCGAATCGAC
3361	AlaAlaIleGlySerValGlyLeuGlyLysValLeuIleAspIleLeuAlaGlyTyrGly GCGCCGCCATCGGCAGTGTTGGACTGGGGAAGGTCCTCATAGACATCCTTGCAGGGTATG CGCGGCGGTAGCCGTCACAACCTGACCCCTTCCAGGAGTATCTGTAGGAACGTCCCATAC
3421	AlaGlyValAlaGlyAlaLeuValAlaPheLysIleMetSerGlyGluValProSerThr GCGCGGGCGTGGCGGAGCTCTTGTGGCATTCAAGATCATGAGCGGTGAGGTCCCCTCCA CGCGCCCGCACCGCCCTCGAGAACACCGTAAGTTCTAGTACTCGCCACTCCAGGGGAGGT
3481	GluAspLeuValAsnLeuLeuProAlaIleLeuSerProGlyAlaLeuValValGlyVal CGGAGGACCTGGTCAATCTACTGCCCGCCATCCTCTCGCCCGGAGCCCTCGTAGTCGGCG GCCTCCTGGACCAGTTAGATGACGGGCGGTAGGAGAGCGGGCCTCGGGAGCATCAGCCGC
3541	ValCysAlaAlaIleLeuArgArgHisValGlyProGlyGluGlyAlaValGlnTrpMet TGGTCTGTGCAGCAATACTGCGCCGGCACGTTGGCCCGGGCGAGGGGGCAGTGCAGTGGA ACCAGACACGTCGTTATGACGCGGCCCGTGCAACCGGGCCCGCTCCCCCGTCACGTCACCT
3601	AsnArgLeulleAlaPheAlaSerArgGlyAsnHisValSerProThrHisTyrValPro TGAACCGGCTGATAGCCTTCGCCTCCCGGGGGAACCATGTTTCCCCCACGCACTACGTGC ACTTGGCCGACTATCGGAAGCGGAGGCCCCCCTTGGTACAAAGGGGGTGCGTGATGCACG
3661	GluSerAspAlaAlaAlaArgValThrAlaIleLeuSerSerLeuThrValThrGlnLeu CGGAGAGCGATGCAGCTGCCCGCGTCACTGCCATACTCAGCAGCCTCACTGTAACCCAGC GCCTCTCGCTACGTCGACGGCGCAGTGACGGTATGAGTCGTCGGAGTGACATTGGGTCG
3721	AGGACTCCGCTGACGTGGTCACCTATTCGAGCCTCACATGGTGAGGTACGAGGCCAAGGA
3781	CCGATTCCCTGTAGACCCTGACCTATACGCTCCACAACTCGCTGAAATTCTGGACCGA11
3841	AlaLysLeuMetProGlnLeuProGlylleProPheValSerCysGlnArgGlyTyrLys AAGCTAAGCTCATGCCACAGCTGCCTGGGATCCCCTTTGTGTCCTGCCAGCGCGGGTATA TTCGATTCGA

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FIG. 26-4

	20,03
3901	GlyValTrpArgValAspGlyIleMetHisThrArgCysHisCysGlyAlaGluIleThr AGGGGGTCTGGCGAGTGGACGGCATCATGCACACTCGCTGCCACTGTGGAGCTGAGATCA TCCCCCAGACCGCTCACCTGCCGTAGTACGTGTGAGCGACGGTGACACCTCGACTCTAGT
3961	GlyHisValLysAsnGlyThrMetArgIleValGlyProArgThrCysArgAsnMetTrr CTGGACATGTCAAAAACGGGACGATGAGGATCGTCGTCCTAGGACCTGCAGGAACATGT GACCTGTACAGTTTTTGCCCTGCTACTCCTAGCAGCCAGGATCCTGGACGTCCTTGTACA
4021	SerGlyThrPheProIleAsnAlaTyrThrThrGlyProCysThrProLeuProAlaProGGAGTGGGACCTTCCCCATTAATGCCTACACCACGGGCCCCTGTACCCCCCTTCCTGCGCCCCTCACCCTGGAAGGGGTAATTACGGATGTGGTGCCCGGGGACATGGGGGGAAGGACGCG
4081	AsnTyrThrPheAlaLeuTrpArgValSerAlaGluGluTyrValGluIleArgGlnValCGAACTACACGTTCGCGCTATGGAGGGTGTCTGCAGAGGAATATGTGGAGATAAGGCAGGGCTTTGATGTGCAAGCGCGATACCTCCCACAGACGTCTCCTTATACACCTCTATTCCGTCC
4141	GlyAspPheHisTyrValThrGlyMetThrThrAspAsnLeuLysCysProCysGlnValTGGGGGACTTCCACTACGTGACGGGTATGACTACTGACAATCTCAAATGCCCGTGCCAGGACCCCCTGAAGGTGATGACTGCCCATACTGATGACTGTTAGAGTTTACGGGCACGGTCC
4201	ProSerProGluPhePheThrGluLeuAspGlyValArgLeuHisArgPheAlaProProTCCCATCGCCCGAATTTTCACAGAATTGGACGGGGTGCGCCTACATAGGTTTGCGCCCCAGGGTAGCGTAGCGTATCCAAAAGGTGTCTTAACCTGCCCCACGCGGATGTATCCAAACGCGGGG
4261	CysLysProLeuLeuArgGluGluValSerPheArgValGlyLeuHisGluTyrProVal CCTGCAAGCCCTTGCTGCGGGAGGAGGTATCATTCAGAGTAGGACTCCACGAATACCCGG GGACGTTCGGGAACGACGCCCTCCTCCATAGTAAGTCTCATCCTGAGGTGCTTATGGGCC
4321	GlySerGlnLeuProCysGluProGluProAspValAlaValLeuThrSerMetLeuThr TAGGGTCGCAATTACCTTGCGAGCCCGAACCGGACGTGGCCGTGTTGACGTCCATGCTCA ATCCCAGCGTTAATGGAACGCTCGGGCTTGGCCTGCACCGGCACAACTGCAGGTACGAGT
4381	AspProSerHisIleThrAlaGluAlaAlaGlyArgArgLeuAlaArgGlySerProProCTGATCCCTCCCATATAACAGCAGAGGCGGCCGGGCGAAGGTTGGCGAGGGGATCACCCCGACTAGGGGAGGTATATTGTCGTCTCCGCCGGCCCGCTTCCAACCGCTCCCTAGTGGGG
4441	Servalalaserserseralaserginleuseralaproserleulysalathrcysthicctctgtggccagctcctcggctagccagctatccgctccatctctctaaggcaacttgcaggaagacaccggtcgagcagctagagagag
4501	AlaAsnHisAspSerProAspAlaGluLeuIleGluAlaAsnLeuLeuTrpArgGlnGluCCGCTAACCATGACTCCCTGATGCTGAGCTCATAGAGGCCAACCTCCTATGGAGGCAGGGGGGGG
4561	MetGlyGlyAsnIleThrArgValGluSerGluAsnLysValVallleLeuAspSerPhe AGATGGGCGGCAACATCACCAGGGTTGAGTCAGAAAACAAAGTGGTGATTCTGGACTCCT TCTACCCGCCGTTGTAGTGGTCCCAACTCAGTCTTTTGTTTCACCACTAAGACCTGAGGA
4621	AspProLeuValAlaGluGluAspGluArgGluIleSerValProAlaGluIleLeuArg TCGATCCGCTTGTGGCGGAGGAGGACGAGCGGGAGATCTCCGTACCCGCAGAAATCCTGC AGCTAGGCGAACACCGCCTCCTCCTGCTCGCCCTCTAGAGGCATGGGCGTCTTTAGGACG
4681	LysSerArgArgPheAlaGlnAlaLeuProValTrpAlaArgProAspTyrAsnProProGGAAGTCTCGGAGATTCGCCCAGGCCCTGCCCGTTTGGGCGCGGCCGGACTATAACCCCCCCC
4741	LeuValGluThrTrpLysLysProAspTyrGluProProValValHisGlyCysProLevCGCTAGTGGAGACGTGGAAAAAGCCCGACTACGAACCACCTGTGGTCCATGGCTGTCCGCGCGCTATCGATCACCTCTGCACCTTTTTCGGGCTGATGCTTGGTGGACACCAGGTACCGACAGGCG
4801	ProproproLysSerProproValProproproArgLysLysArgThrValValLeuTh: TTCCACCTCCAAAGTCCCCTCCTGTGCCTCCGCCTCGGAAGAAGCGGACGGTGGTCCTCA AAGGTGGAGGTTTCAGGGGAGGACACGGAGGCGGAGCCTTCTTCGCCTGCCACCAGGAGT
	GluSerThrLeuSerThrAlaLeuAlaGluLeuAlaThrArgSerPheGlySerSerSe

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FIG. 26-5

4861	CTGAATCAACCCTATCTACTGCCTTGGCCGAGCTCGCCACCAGAAGCTTTGGCAGCTCCT GACTTAGTTGGGATAGATGACGGAACCGGCTCGAGCGTGGTCTTCGAAACCGTCGAGGA
4921	ThrSerGlyIleThrGlyAspAsnThrThrThrSerSerGluProAlaProSerGlyCys CAACTTCCGGCATTACGGGCGACAATACGACAACATCCTCTGAGCCCGCCC
4981	ProProAspSerAspAlaGluSerTyrSerSerMetProProLeuGluGlyGluProGlyGCCCCCCGACTCCGACGCTGAGTCCTATTCCTCCATGCCCCCCTGGAGGGGGAGCCTGCGGGGGGGG
5041	AspProAspLeuSerAspGlySerTrpSerThrValSerSerGluAlaAsnAlaGluAsp GGGATCCGGATCTTAGCGACGGGTCATGGTCAACGGTCAGTAGTGAGGCCAACGCGGAGG CCCTAGGCCTAGAATCGCTGCCCAGTACCAGTTGCCAGTCATCACTCCGGTTGCGCCTCC
5101	ValValCysCysSerMetSerTyrSerTrpThrGlyAlaLeuValThrProCysAlaAla ATGTCGTGTGCTCAATGTCTTACTCTTGGACAGGCGCACTCGTCACCCCGTGCGCCG TACAGCACACGACGAGTTACAGAATGAGAACCTGTCCGCGTGAGCAGTGGGGCACGCGGC
5161	GlucluGlnLysLeuProlleAsnAlaLeuSerAsnSerLeuLeuArgHisHisAsnLeuCGGAAGAACCAAATGCACTAAGCAACTCGTTGCTACGTCACCACAATTGCCTTCTTGTCTTCTCTCTC
5221	ValtyrSerThrThrSerArg_ TGGTGTATTCCACCACCTCACGCAGTGCACCGCACGGCACGGCAGAGAAAGTCACATTTGACA ACCACATAAGGTGGTGGAGTGCGTCACGAACGGCAGAGAAAGTCACATTTGACA
5281	LeuGlnValleuAspSerHisTyrGlnAspValleuLysGluValleuAspSerHisTyrGlnAspValleuAspSerHisTyrGlnAspValleuAspSerHisTyrGlnAspValleuAspSerHisTyrGlnAspValleuAspSerHisTyrGlnAspValleuAspSerHisTyrGlnAspValleuAspSerHisTyrGlnAspCoxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
5341	LysValLysAlaAsnLeu CAAAAGTGAAGGCTAACTTG GTTTTCACTTCCGATTGAAC

FIG. 26-6

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F	IG. 27	Translation	of DNA	12f	
1	IlePhel CCATATTT GGTATAAA	LyslleArgMet AAAATCAGGATG PTTTAGTCCTAC	TyrValGl TACGTGGG ATGCACCC	yGlyValGluHisArg AGGGGTCGAACACAGG TCCCCAGCTTGTGTCC	LeuGluAlaAlaCysAsr CTGGAAGCTGCCTGCA GACCTTCGACGGACGT
61	ACTICATOR	CCCCCCAACCT	TGCGATCT	uGluaspargasparg GGAAGACAGGGACAGG CCTTCTGTCCCTGTCC	SerGluLeuSerProLeu ICCGAGCTCAGCCCGT AGGCTCGAGTCGGGCA
121	TACTGCTG	CCACTACACAG	rggcaggi	lleuprocysserphe CCTCCCGTGTTCCTTC GGAGGGCACAAGGAAG	ThrThrLeuProAlaLeu ACAACCCTACCAGCCT IGTTGGGATGGTCGGA
181	TGTCCACC	GCCTCATCCAC	CTCCACCA	nAsnIleValAspVal(GAACATTGTGGACGTG(CTTGTAACACCTGCAC(SintyrLeutyrGlyVal LAGTACTTGTACGGG STCATGAACATGCCCC
241	TGGGGTCAZ	GCATCGCGTCC	IGGGCCAT	eLysTrpGluTyrVal\ TAAGTGGGAGTACGTC(ATTCACCCTCATGCAG(ValleuLeuPheLeuLeu GTTCTCCTGTTCCTTC CAAGAGGACAAGGAAG
301	TGCTTGCAC	ACGCGCGCGTC	rgctcctg	SLeuTrpMetMetLeuI CTTGTGGATGATGCTA(GAACACCTACTACGAT(LeulleSerGlnAlaGlu CTCATATCCCAAGCGG SAGTATAGGGTTCGCC
361	AGGCGGCT	LeuGluAsnLeu\ TTGGAGAACCTC	/allleLe STAATACT	14iuAsnAlaAlaSerLew TAATGCAGCATCCCTGC ATTACGTCGTAGGGACC	AlaGlyThrHisGlyLeu SCCGGGACGCACGGTC CGGCCCTGCGTGCCAG
421	Val TTGTATC AACATAG			•	

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FIG. 28 Translation of DNA 35f

- LeuLysGluValLysAlaAlaAlaSerLysValLysAlaAsnLeuLeuSerValGluGlu
 TGCTCAAGGAGGTTAAAGCAGCGGCGTCAAAAGTGAAGGCTAACTTGCTATCCGTAGAGG
 ACGAGTTCCTCCAATTTCGTCGCCGCAGTTTTCACTTCCGATTGAACGATAGGCATCTCC
- AlaCysSerLeuThrProProHisSerAlaLysSerLysPheGlyTyrGlyAlaLysAsp
 61 AAGCTTGCAGCCTGACGCCCCCACACTCAGCCAAATCCAAGTTTGGTTATGGGGCAAAAG
 TTCGAACGTCGGACTGCGGGGGTGTGAGTCGGTTTAGGTTCAAACCAATACCCCGTTTTC
- GluAspAsnValThrProIleAspThrThrIleMetAlaLysAsnGluValPheCysVal 181 TGGAAGACAATGTAACACCAATAGACACTACCATCATGGCTAAGAACGAGGTTTTCTGCG ACCTTCTGTTACATTGTGGTTATCTGTGATGGTAGTACCGATTCTTGCTCCAAAAGACGC
- GlnProGluLysGlyGlyArgLysProAlaArgLeuIleValPheProAspLeuGlyVal TTCAGCCTGAGAAGGGGGTCGTAAGCCAGCTCGTCTCATCGTGTTCCCCGATCTGGGCG AAGTCGGACTCTTCCCCCCAGCATTCGGTCGAGCAGAGTAGCACAAGGGGCTAGACCCGC
- ArgValCysGluLysMetAlaLeuTyrAspValValThrLysLeuProLeuAlaValMet
 TGCGCGTGTGCGAAAAGATGGCTTTGTACGACGTGGTTACAAAGCTCCCCTTGGCCGTGA
 ACGCGCACACGCTTTTCTACCGAAACATGCTGCACCAATGTTTCGAGGGGAACCGGCACT
- GlySerSerTyrGlyPheGlnTyrSerProGlyGlnArgValGluPheLeuValGlnAla 361 TGGGAAGCTCCTACGGATTCCAATACTCACCAGGACAGCGGGTTGAATTCCTCGTGCAAG ACCCTTCGAGGATGCCTAAGGTTATGAGTGGTCCTGTCGCCCAACTTAAGGAGCACGTTC
- TrpLysSerLysLysThrProMetGlyPheSerTyrAspThrArgCysPheAspSerThr 421 CGTGGAAGTCCAAGAAAACCCCAATGGGGTTCTCGTATGATACCCGCTGCTTTGACTCCA GCACCTTCAGGTTCTTTTGGGGTTACCCCAAGAGCATACTATGGGCGACGAAACTGAGGT
- ValThrGluSerAspIleArgThrGluGluAla
 481 CAGTCACTGAGAGCGACATCCGTACGGAGGAGGCA
 GTCAGTGACTCTCGCTGTAGGCATGCCTCCTCCGT

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FIG. 29 Translation of DNA 19g

 ${\tt GluPheLeuValGlnAlaTrpLysSerLysLysThrProMetGlyPheSerTyrAspThr}$ GAATTCCTCGTGCAAGCGTGGAAGTCCAAGAAAACCCCAATGGGGTTCTCGTATGATACC CTTAAGGAGCACGTTCGCACCTTCAGGTTCTTTTGGGGTTACCCCAAGAGCATACTATGG ----Overlap with 35f--- ${\tt ArgCysPheAspSerThrValThrGluSerAspIleArgThrGluGluAlaIleTyrGln}$ CGCTGCTTTGACTCCACAGTCACTGAGAGCGACATCCGTACGGAGGAGGCAATCTACCAA GCGACGAAACTGAGGTGTCAGTGACTCTCGCTGTAGGCATGCCTCCTCCGTTAGATGGTT ${\tt CysCysAspLeuAspProGlnAlaArgValAlaIleLysSerLeuThrGluArgLeuTyr}$ TGTTGTGACCTCGACCCCCAAGCCCGCGTGGCCATCAAGTCCCTCACCGAGAGGCTTTAT ACAACACTGGAGCTGGGGGTTCGGGCGCACCGGTAGTTCAGGGAGTGGCTCTCCGAAATA ValGlyGlyProLeuThrAsnSerArgGlyGluAsnCysGlyTyrArgArgCysArgAla GTTGGGGGCCCTCTTACCAATTCAAGGGGGGAGAACTGCGGCTATCGCAGGTGCCGCGC 181 CAACCCCGGGAGAATGGTTAAGTTCCCCCCTCTTGACGCCGATAGCGTCCACGGCGCGC SerGlyValLeuThrThrSerCysGlyAsnThrLeuThrCysTyrIleLysAlaArgAla AGCGGCGTACTGACAACTAGCTGTGGTAACACCCTCACTTGCTACATCAAGGCCCGGGCA TCGCCGCATGACTGTTGATCGACACCATTGTGGGAGTGAACGATGTAGTTCCGGGCCCGT ${\tt AlaCysArgAlaAlaGlyLeuGlnAspCysThrMetLeuValCysGlyAspAspLeuVal}$ GCCTGTCGAGCCGCAGGGCTCCAGGACTGCACCATGCTCGTGTGTGGCGACGACTTAGTC CGGACAGCTCGGCGTCCCGAGGTCCTGACGTGGTACGAGCACACCCGCTGCTGAATCAG ValIleCysGluSerAlaGlyValGlnGluAspAlaAla 361 GTTATCTGTGAAAGCGCGGGGTCCAGGAGGACGCGGCGAG CAATAGACACTTTCGCGCCCCCAGGTCCTCCTGCGCCGCTC

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FIG. 30 Translation of DNA 26g

- GlyGlyGluAsnCysGlyTyrArgArgCysArgAlaSerGlyValLeuThrThrSerCys
 GGGGGGGAGAACTGCGGCTATCGCAGGTGCCGCGCAAGCGGCGTACTGACAACTAGCTGT
 CCCCCCCTCTTGACGCCCGATAGCGTCCACGGCGCGTTCGCCGCATGACTGTTGATCGACA
- GlyAsnThrLeuThrCysTyrIleLysAlaArgAlaAlaCysArgAlaAlaGlyLeuGln
 61 GGTAACACCCTCACTTGTTACATCAAGGCCCGAGCAGCCTGTCGAGCCGCAGGGCTCCAG
 CCATTGTGGGAGTGAACAATGTAGTTCCGGGCTCGTCGGACAGCTCGGCGTCCCGAGGTC
- GlnGluAspAlaAlaSerLeuArgAlaPheThrGluAlaMetThrArgTyrSerAlaPro
 181 CAGGAGGACGCGGCGAGCCTGAGAGCCTTCACGGAGGCTATGACCAGGTACTCCGCCCCC
 GTCCTCCTGCGCCCCCTCGGACTCTCGGAAGTGCCTCCGATACTGGTCCATGAGGCGGGG
- ProGlyAspProProGlnProGluTyrAspLeuGluLeuIleThrSerCysSerSerAsn
 CCTGGGGACCCCCACAACCAGAATACGACTTGGAGCTCATAACATCATGCTCCCAAC
 GGACCCCTGGGGGGTGTTGGTCTTATGCTGAACCTCGAGTATTGTAGTACGAGGAGGTTG
- ValSerValAlaHisAspGlyAlaGlyLysArgValTyrTyrLeuThrArgAspProThr 301 GTGTCAGTCGCCCACGACGGCGCTGGAAAGAGGGTCTACTACCTCACCCGTGACCCTACA CACAGTCAGCGGGTGCTGCCGCACCTTTCTCCCAGATGATGGAGTGGCACTGGGATGT
- ThrProLeuAlaArgAlaAlaTrpGluThrAlaArgHisThrProValAsnSerTrpLeu
 361 ACCCCCTCGCGAGAGCTGCGTGGGAGACACCACACACACTCCAGTCAATTCCTGGCTA
 TGGGGGGAGCGCTCTCGACGCACCCTCTGTCGTTCTGTGTGAGGTCAGTTAAGGACCGAT
- GlyAsnIleIleMetPheAlaProThrLeuTrpAla 421 GGCAACATAATCATGTTTGCCCCCACACTGTGGGCG CCGTTGTATTAGTACAAACGGGGGTGTGACACCCGC

FIG. 3 | Translation of DNA 15e

- GlyAlaGlyLysArgValTyrTyrLeuThrArgAspProThrThrProLeuAlaArgAla
 CGGCGCTGGAAAGAGGGTCTACTACCTCACCCGTGACCCTACAACCCCCCTTCGCGAGAGC
 GCCGCGACCTTTCTCCCCAGATGATGGAGTGGGCACTGGGATGTTGGGGGGAGCGCTCTCG
- AlaTrpGluThrAlaArgHisThrProValAsnSerTrpLeuGlyAsnIleIleMetPhe
 TGCGTGGGAGACAGCAAGACACCTCCAGTCAATTCCTGGCTAGGCAACATAATCATGTT
 ACGCACCCTCTGTCGTTCTGTGTGAGGTCAGTTAAGGACCGATCCGTTGTATTAGTACAA
- AlaProThrLeuTrpAlaArgMetIleLeuMetThrHisPhePheSerValLeuIleAla TGCCCCCACACTGTGGGCGAGGATGATACTGATGACCCATTTCTTTAGCGTCCTTATAGC ACGGGGGTGTGACACCCGCTCCTACTATGACTACTGGGTAAAGAAATCGCAGGAATATCG
- ArgAspGlnLeuGluGlnAlaLeuAspCysGluIleTyrGlyAlaCysTyrSerlleGlu
 181 CAGGGACCAGCTTGAACAGGCCCTCGATTGCGAGATCTACGGGGCCTGCTACTCCATAGA
 GTCCCTGGTCGAACTTGTCCGGGAGCTAACGCTCTAGATGCCCCGGACGATGAGGTATCT
- ProLeuAspLeuProProIleIleGlnArgLeu
 241 ACCACTTGATCTACCTCCAATCATTCAAAGACTC
 TGGTGAACTAGATGGAGGTTAGTAAGTTTCTGAG

AUDOTITUTE CUEST

FIG. 32-1 COMBINED ORF OF DNAs 12f through 15e

- TrpThrArgGlyGluArgCysAspLeuGluAspArgAspArgSerGluLeuSerProLeu
 61 ACTGGACGCGGGCGAACGTTGCGATCTGGAAGACAGGGACAGGTCCGAGCTCAGCCCGT
 TGACCTGCGCCCCGCTTGCAACGCTAGACCTTCTGTCCCTGTCCAGGCTCGAGTCGGGCA
- LeuLeuThrThrThrGlnTrpGlnValLeuProCysSerPheThrThrLeuProAlaLeu
 121 TACTGCTGACCACTACACAGTGGCAGGTCCTCCCGTGTTCCTTCACAACCCTACCAGCCT
 ATGACGACTGGTGATGTCACCGTCCAGGAGGGCACAAGGAAGTGTTGGGATGGTCGGA
- SerThrGlyLeuIleHisLeuHisGlnAsnIleValAspValGlnTyrLeuTyrGlyVal
 181 TGTCCACCGGCCTCATCCACCTCACCAGAACATTGTGGACGTGCAGTACTTGTACGGGG
 ACAGGTGGCCGGAGTAGGTGGAGGTGGTCTTGTAACACCTGCACGTCATGAACATGCCCC
- GlySerSerIleAlaSerTrpAlaIleLysTrpGluTyrValValLeuLeuPheLeuLeu

 TGGGGTCAAGCATCGCGTCCTGGGCCATTAAGTGGGAGTACGTCGTTCCTTGTTCCTTC

 ACCCCAGTTCGTAGCGCAGGACCCGGTAATTCACCCTCATGCAGCAAGAGGACAAGGAAG
- AlaAlaLeuGluAsnLeuValIleLeuAsnAlaAlaSerLeuAlaGlyThrHisGlyLeu
 361 AGGCGGCTTTGGAGAACCTCGTAATACTTAATGCAGCATCCCTGGCCGGACGCACGGTC
 TCCGCCGAAACCTCTTGGAGCATTATGAATTACGTCGTAGGGACCGGCCCTGCGTGCCAG
- ValSerPheLeuValPhePheCysPheAlaTrpTyrLeuLysGlyLysTrpValProGly
 TTGTATCCTTCCTCGTGTTCTTCTGCTTTGCATGGTATTTGAAGGGTAAGTGGGTGCCCG
 AACATAGGAAGGAGCACAAGAAGACGAAACGTACCATAAACTTCCCATTCACCCACGGGC
- AlaValTyrThrPheTyrGlyMetTrpProLeuLeuLeuLeuLeuLeuLeuAlaLeuProGln
 GAGCGGTCTACACCTTCTACGGGATGTGGCCTCTCCTCCTGCTCCTGTTGGCGTTGCCCC
 CTCGCCAGATGTGGAAGATGCCCTACACCGGAGAGGAGGACGACGACAACCGCAACGGGG
- LeuMetAlaLeuThrLeuSerProTyrTyrLysArgTyrIleSerTrpCysLeuTrpTrp
 601 GGTTGATGGCGCTGACTCTGTCACCATATTACAAGCGCTATATCAGCTGGTGCTTGTGGT
 CCAACTACCGCGACTGAGACAGTGGTATAATGTTCGCGATATAGTCGACCACGAACACCA
- LeuGlnTyrPheLeuThrArgValGluAlaGlnLeuHisValTrpIleProProLeuAsn
 661 GGCTTCAGTATTTTCTGACCAGAGTGGAAGCGCAACTGCACGTGTGGATTCCCCCCCTCA
 CCGAAGTCATAAAAGACTGGTCTCACCTTCGCGTTGACGTGCACACCTAAGGGGGGGAGT
- ValargGlyGlyArgAspAlaValIleLeuLeuMetCysAlaValHisProThrLeuVal
 ACGTCCGAGGGGGGCGCGCGCGTCATCTTACTCATGTGTGCTGTACACCCGACTCTGG
 TGCAGGCTCCCCCCGCGCTGCGCAGTAGAATGAGTACACACGACATGTGGGCTGAGACC
- PheAspIleThrLysLeuLeuAlaValPheGlyProLeuTrpIleLeuGlnAlaSer
 781 TATTTGACATCACCAAATTGCTGCTGGCCGTCTTCGGACCCCTTTGGATTCTTCAAGCCA
 ATAAACTGTAGTGGTTTAACGACGACCGGCAGAAGCCTGGGGAAACCTAAGAAGTTCGGT
- LeuLeuLysValProTyrPheValArgValGlnGlyLeuLeuArgPheCysAlaLeuAla 841 GTTTGCTTAAAGTACCCTACTTTGTGCGCGTCCAAGGCCTTCTCCGGTTCTGCGCGTTAG CAAACGAATTTCATGGGATGAAACACGCGCAGGTTCCGGAAGAGGCCAAGACGCGCAATC
- ArgLysMetlleGlyGlyHisTyrValGlnMetVallleIleLysLeuGlyAlaLeuThr CGCGGAAGATGATCGGAGGCCATTACGTGCAAATGGTCATCATTAAGTTAGGGGCGCTTA GCGCCTTCTACTAGCCTCCGGTAATGCACGTTTACCAGTAGTAATTCAATCCCCGCGAAT

SUBSTITUTE SHEET

961	GlyThrTyrValTyrAsnHisLeuThrProLeuArgAspTrpAlaHisAsnGlyLeuArg CTGGCACCTATGTTTATAACCATCTCACTCCTCTTCGGGACTGGGCGCACAACGGCTTGC GACCGTGGATACAAATATTGGTAGAGTGAGGAGAAGCCCTGACCCGCGTGTTGCCGAACG
1021	AspLeuAlaValAlaValGluProValValPheSerGlnMetGluThrLysLeuIleThr GAGATCTGGCCGTGGCTGTAGAGCCAGTCGTCTTCTCCCAAATGGAGACCAAGCTCATCA CTCTAGACCGGCACCGACATCTCGGTCAGCAGAAGAGGGTTTACCTCTGGTTCGAGTAGT
1081	TrpGlyAlaAspThrAlaAlaCysGlyAspIleIleAsnGlyLeuProValSerAlaArg CGTGGGGGGCAGATACCGCCGCGTGCGTGACATCATCAACGGCTTGCCTGTTTCCGCCC GCACCCCCGTCTATGGCGGCGCACGCCACTGTAGTAGTTGCCGAACGGACAAAGGCGGG
1141	ArgGlyArgGluIleLeuLeuGlyProAlaAspGlyMetValSerLysGlyTrpArgLeu GCAGGGGCCGGAGATACTGCTCGGGCCAGCCGATGGAATGGTCTCCAAGGGGTGGAGGT CGTCCCCGGCCCTCTATGACGAGCCCGGTCGGCTACCTTACCAGAGGTTCCCCACCTCCA
1201	LeuAlaProlleThrAlaTyrAlaGlnGlnThrArgGlyLeuLeuGlyCyslleIleThr TGCTGGCGCCCATCACGGCGTACGCCCAGCAGACAAGGGGCCCTCCTAGGGTGCATAATCA ACGACCGCGGGTAGTGCCGCATGCGGGTCGTCTGTTCCCCGGAGGATCCCACGTATTAGT
1261	SerLeuThrGlyArgAspLysAsnGlnValGluGlyGluValGlnIleValSerThrAla CCAGCCTAACTGGCCGGGACAAAAACCAAGTGGAGGGTGAGGTCCAGATTGTGTCAACTG GGTCGGATTGACCGGCCCTGTTTTTGGTTCACCTCCCACTCCAGGTCTAACACAGTTGAC
1321	AlaGlnThrPheLeuAlaThrCysIleAsnGlyValCysTrpThrValTyrHisGlyAla CTGCCCAAACCTTCCTGGCAACGTGCATCAATGGGGTGTGCTGGACTGTCTACCACGGGG GACGGGTTTGGAAGGACCGTTGCACGTAGTTACCCCCACACGACCTGACAGATGGTGCCCC
1381	GlyThrArgThrIleAlaSerProLysGlyProVallleGlnMetTyrThrAsnValAsp CCGGAACGAGGACCATCGCGTCACCCAAGGGTCCTGTCATCCAGATGTATACCAATGTAG GGCCTTGCTCCTGGTAGCGCAGTGGGTTCCCAGGACAGTAGGTCTACATATGGTTACATC
1441	GlnAspleuValGlyTrpProAlaProGlnGlySerArgSerLeuThrProCysThrCys ACCAAGACCTTGTGGGCTGGCCCGCTCCGCAAGGTAGCCGCTCATTGACACCCTGCACTT TGGTTCTGGAACACCCGACCGGGCGAGGCGTTCCATCGGCGAGTAACTGTGGGACGTGAA
1501	GlySerSerAspLeuTyrLeuValThrArgHisAlaAspValIleProValArgArgArgGCGGGCTCCTCGGACCTTTACCTGGTCACGAGGCACGCCGATGTCATTCCCGTGCGCCGGCCG
1561	GlyAspSerArgGlySerLeuLeuSerProArgProIleSerTyrLeuLysGlySerSer GGGGTGATAGCAGGGGCAGCCTGCTGTCGCCCCGGCCCATTTCCTACTTGAAAGGCTCCT CCCCACTATCGTCCCCGTCGGACGACAGCGGGGCCGGGTAAAGGATGAACTTTCCGAGGA
1621	GlyGlyProLeuLeuCysProAlaGlyHisAlaValGlyIlePheArgAlaAlaValCys CGGGGGGTCCGCTGTTGTGCCCCGCGGGCACGCCGTGGGCATATTTAGGGCCGCGGTGT GCCCCCCAGGCGACAACACGGGGCGCCCCGTGCGGCACCCGTATAAATCCCGGCGCCACA
1681	CGTGGGCACCTCACCGATTCCGCCACCTGAAATAGGGACACCTCTTGGATCTCTGTTGGT
1741	ACTCCAGGGGCCACAAGTGCCTATTGAGGAGAGGTGGTCATCACGGGGTCTCGAAGGTCC
1801	ACCGAGTGGAGGTACGAGGGTGTCCGTCGCCGTTTTCGTGGTTCCAGGGCCGACGTATAC
186]	AlaGlnGlyTyrLysValLeuValLeuAsnProSerValAlaAlaThrLeuGlyPheGly CAGCTCAGGGCTATAAGGTGCTAGTACTCAACCCCTCTGTTGCTGCAACACTGGGCTTTG GTCGAGTCCCGATATTCCACGATCATGAGTTGGGGAGACAACGACGTTGTGACCCGAAAC

 ${\tt AlaTyrMetSerLysAlaHisGlyIleAspProAsnIleArgThrGlyValArgThrIle}$

FIG. 32-2

CHRSTITHTE SHEET

1921	34 / 0 3 GTGCTTACATGTCCAAGGCTCATGGGATCGATCCTAACATCAGGACCGGGGTGAGAACAA. CACGAATGTACAGGTTCCGAGTACCCTAGCTAGGATTGTAGTCCTGGCCCCACTCTTGTT
1981	ThrThrGlySerProlleThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGlyCys TTACCACTGGCAGCCCCATCACGTACTCCACCTACGGCAAGTTCCTTGCCGACGGCGGGT AATGGTGACCGTCGGGGTAGTGCATGAGGTGGATGCCGTTCAAGGAACGGCTGCCGCCCA
2041	SerGlyGlyAlaTyrAspIleIleIleCysAspGluCysHisSerThrAspAlaThrSer GCTCGGGGGGCGCTTATGACATAATAATTTGTGACGAGTGCCACTCCACGGATGCCACAT CGAGCCCCCGGGAATACTGTATTATTAAACACTGCTCACGGTGAGGTGCCTACGGTGTA
2101	IleLeuGlyIleGlyThrValLeuAspGlnAlaGluThrAlaGlyAlaArgLeuValValCCATCTTGGGCATCGGCACTGTTGACCAAGCAGACTGCGGGGCGAGACTGGTTGGT
2161	LeuAlaThrAlaThrProProGlySerValThrValProHisProAsnIleGluGluVal TGCTCGCCACCGCCACCCCTCCGGGCTCCGTCACTGTGCCCCATCCCAACATCGAGGAGG ACGAGCGGTGGCGGTGGGGAGGCCCGAGGCAGTGACACGGGGTAGGGTTGTAGCTCCTCC
2221	AlaLeuSerThrThrGlyGluIleProPheTyrGlyLysAlaIleProLeuGluValIle TTGCTCTGTCCACCACCGGAGAGATCCCTTTTTACGGCAAGGCTATCCCCCTCGAAGTAA AACGAGACAGGTGGTGGCCTCTCTAGGGAAAAATGCCGTTCCGATAGGGGGAGCTTCATT
2281	LysGlyGlyArgHisLeullePheCysHisSerLysLysLysCysAspGluLeuAlaAla TCAAGGGGGGGAGACATCTCATCTTCTGTCATTCAAAGAAGAGTGCGACGAACTCGCCG AGTTCCCCCCCTCTGTAGAGTAGA
2341	LysLeuValAlaLeuGlyIleAsnAlaValAlaTyrTyrArgGlyLeuAspValSerVal CAAAGCTGGTCGCATTGGGCATCAATGCCGTGGCCTACTACCGCGGTCTTGACGTGTCCG GTTTCGACCAGCGTAACCCGTAGTTACGGCACCGGATGATGGCGCCAGAACTGCACAGGC
2401	IleProThrSerGlyAspValValValAlaThrAspAlaLeuMetThrGlyTyrThr TCATCCCGACCAGCGGCGATGTTGTCGTCGTGGCAACCGATGCCCTCATGACCGGCTATA AGTAGGGCTGGTCGCCGCTACAACAGCAGCACCGTTGGCTACGGGAGTACTGGCCGATAT
2461	GlyAspPheAspSerVallleAspCysAsnThrCysValThrGlnThrValAspPheSer CCGGCGACTTCGACTCGGTGATAGACTGCAATACGTGTCACCCAGACAGTCGATTTCA GGCCGCTGAAGCTGAGCCACTATCTGACGTTATGCACACAGTGGGTCTGTCAGCTAAAGT
2521	LeuAspProThrPheThrlleGluThrlleThrLeuProGlnAspAlaValSerArgThr GCCTTGACCCTACCTTCACCATTGAGACAATCACGCTCCCCAGGATGCTGTCTCCCGCA CGGAACTGGGATGGAAGTGGTAACTCTGTTAGTGCGAGGGGGTCCTACGACAGAGGGCGT
2581	GlnArgArgGlyArgThrGlyArgGlyLysProGlyIleTyrArgPheValAlaProGlyCTCAACGTCGGGGCAGGACTGGCAGGGGGAAGCCAGGCATCTACAGATTTGTGGCACCGGGAGTTTGCAGCCCCGTCCGT
2641	GluArgProSerGlyMetPheAspSerSerValLeuCysGluCysTyrAspAlaGlyCys GGGAGCGCCCTCCGGCATGTTCGACTCGTCCGTCCTGTGAGTGCTATGACGCAGGCT CCCTCGCGGGGAGGCCGTACAAGCTGAGCAGGCAGGAGACACTCACGATACTGCGTCCGA
2701	AlaTrpTyrGluLeuThrProAlaGluThrThrValArgLeuArgAlaTyrMetAsnThr GTGCTTGGTATGAGCTCACGCCCGCCGAGACTACAGTTAGGCTACGAGCGTACATGAACA CACGAACCATACTCGAGTGCGGGCGGCTCTGATGTCAATCCGATGCTCGCATGTACTTGT
2761	ProGlyLeuProValCysGlnAspHisLeuGluPheTrpGluGlyValPheThrGlyLeuCCCCGGGGCTTCCCGTGTGCCAGGACCATCTTGAATTTTGGGAGGGCGTCTTTACAGGCCGGGGCCCCGAAGGGCACACGGTCCTGGTAGAACCTTAAAACCCTCCCGCAGAAATGTCCGG
2821	ThrHisIleAspAlaHisPheLeuSerGlnThrLysGlnSerGlyGluAsnLeuProTyr TCACTCATATAGATGCCCACTTTCTATCCCAGACAAAGCAGAGTGGGGAGAACCTTCCTT
2881	LeuValAlaTyrGlnAlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAsp ACCTGGTAGCGTACCAAGCCACCGTGTGCGCTAGGGCTCAAGCCCCTCCCCCATCGTGGG TGGACCATCGCATGGTTCGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGGTAGCACCC

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CHRCTITHITF SHEET

FIG. 32-3

	35/63
2941	35/63 GlnMetTrpLysCysLeuIleArgLeuLysProThrLeuHisGlyProThrProLeuLeu ACCAGATGTGGAAGTGTTTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGC TGGTCTACACCTTCACAAACTAAGCGGAGTTCGGGTGGGAGGTACCCCGGTTGTGGGGACG
3001	TyrArgLeuGlyAlaValGlnAsnGluIleThrLeuThrHisProValThrLysTyrIle TATACAGACTGGGCGCTGTTCAGAATGAAATCACCCTGACGCACCCAGTCACCAAATACA ATATGTCTGACCCGCGACAAGTCTTACTTTAGTGGGACTGCGTGGGTCAGTGGTTTATGT
3061	MetThrCysMetSerAlaAspLeuGluValValThrSerThrTrpValLeuValGlyGly TCATGACATGCATGTCGGCCGACCTGGAGGTCGTCACGAGCACCTGGGTGCTCGTTGGCG AGTACTGTACGTACAGCCGGCTGGACCTCCAGCAGTGCTCGTGGACCCACGAGCAACCGC
3121	ValleuAlaAlaLeuAlaAlaTyrCysLeuSerThrGlyCysValValIleValGlyArg GCGTCCTGGCTGTTTGGCCGCGTATTGCCTGTCAACAGGCTGCGTGGTCATAGTGGGCA CGCAGGACCGACGAAACCGGCGCATAACGGACAGTTGTCCGACGACCAGTATCACCCGT
3181	ValValLeuSerGlyLysProAlaIleIleProAspArgGluValLeuTyrArgGluPhe GGGTCGTCTTGTCCGGGAAGCCGGCAATCATACCTGACAGGAAGTCCTCTACCGAGAGT CCCAGCAGAACAGGCCCTTCGGCCGTTAGTATGGACTGTCCCTTCAGGAGATGGCTCTCA
3241	AspGluMetGluGluCysSerGlnHisLeuProTyrIleGluGlnGlyMetMetLeuAla TCGATGAGATGGAAGAGTGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCTCG AGCTACTCTACCTTCTCACGAGAGTCGTGAATGGCATGTAGCTCGTTCCCTACTACGAGC
3301	GluGlnPheLysGlnLysAlaLeuGlyLeuLeuGlnThrAlaSerArgGlnAlaGluVal CCGAGCAGTTCAAGCAGAAGGCCCTCGGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGG GGCTCGTCAAGTTCGTCTTCCGGGAGCCGGAGGACGTCTGGCGCAGGGCAGTCCGTCTCC
3361	IleAlaProAlaValGlnThrAsnTrpGlnLysLeuGluThrPheTrpAlaLysHisMet TTATCGCCCCTGCTGTCCAGACCAACTGGCAAAAACTCGAGACCTTCTGGGCGAAGCATA AATAGCGGGGACGACAGGTCTGGTTGACCGTTTTTGAGCTCTGGAAGACCCGCTTCGTAT
3421	TrpAsnPheIleSerGlyIleGlnTyrLeuAlaGlyLeuSerThrLeuProGlyAsnPro TGTGGAACTTCATCAGTGGGATACAATACTTGGCGGGCTTGTCAACGCTGCCTGGTAACC ACACCTTGAAGTAGTCACCCTATGTTATGAACCGCCCGAACAGTTGCGACGGACCATTGG
3481	AlailealaSerLeuMetAlaPheThralaAlaValThrSerProLeuThrThrSerGln CCGCCATTGCTTCATTGATGGCTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCC GGCGGTAACGAAGTAACTACCGAAAATGTCGACGACAGTGGTCGGGTGATTGGTGATCGG
3541	ThrLeuLeuPheAsnIleLeuGlyGlyTrpValAlaAlaGlnLeuAlaAlaProGlyAla AAACCCTCCTCTTCAACATATTGGGGGGGGTGGGTGGCTGCCCAGCTCGCCGCCCCCGGTG TTTGGGAGGAGAAGTTGTATAACCCCCCCACCCACCGACGGGTCGAGCGGGGGGGCCAC
3601	AlaThrAlaPheValGlyAlaGlyLeuAlaGlyAlaAlaIleGlySerValGlyLeuGly CCGCTACTGCCTTTGTGGGCGCTGGCTTAGCTGGCCGCCGCCATCGGCAGTGTTGGACTGG GGCGATGACGGAAACACCCGCGACCGAATCGACCGCGGCGGTAGCCGTCACAACCTGACC
3661	CCTTCCAGGAGTATCTGTAGGAACGTCCCATACCGCGCCCGCACCGCCCTCGAGAACACC
3721	GTAAGTTCTAGTACTCGCCACTCCAGGGGAGGTGCCTCCTGGACCAGTTAGTT
3781	GGTAGGAGAGCGGCCTCGGGAGCATCAGCCGCACCAGACACGTCGTIMTONOGGGGCGCATCAGCCGCACCAGACACGTCGTIMTONOGGGGCGCATCAGCCGCAGACACGTCGTIMTONOGGGGCGCAGCAGACACGTCGTIMTONOGGGGCGCAGCAGACACGTCGGTAGACACGGTCGGT
3841	ValGlyProGlyGluGlyAlaValGlnTrpMetAsnArgLeuIleAlaPheAlaSerArg ACGTTGGCCCGGGCGAGGGGGCAGTGCAGTGGATGACCTGCTGATAGCCTTCGCCTCCC ACGTTGGCCCGACTATCGCCGAAGCGGAGGG

 ${\tt GlyAsnHisValSerProThrHisTyrValProGluSerAspAlaAlaAlaArgValThr}$

FIG. 32-4

CHRCTITHE SHEET

FIG.	32-5
4861	SerGluAsnLysValValIleLeuAspSerPheAspProLeuValAlaGluGluAspGluAspGluAsnCAAAACAAAGTGGTGATTCTGGACTCCTTCGATCCGCTTGTGGCGGAGGAGGACGTCAGTCTTTTGTTTCACCACTAAGACCTGAGGAAGCTAGGCGAACACCGCCTCCTCCTGC
4801	LeulleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGlyAsnIleThrArgValGlyAGCTCATAGAGGCCAACCTCCTATGGAGGCAGGAGATGGGCGGCAACATCACCAGGGTTGTCGAGTATCTCCGGTTGGAGGATACCTCCGTCCTCTACCCGCCGTTGTAGTGGTCCCAAC
4741	LeuSerAlaProSerLeuLysAlaThrCysThrAlaAsnHisAspSerProAspAlaGluaGCTATCCGCTCCATCTCTCAAGGCAACTTGCACCGCTAACCATGACTCCCCTGATGCTGTCGATAGGCGAGGGTAGAGAGTTCCGTTGAACGTGGCGATTGGTACTGAGGGGACTACGAC
4681	AlaGlyArgArgLeuAlaArgGlySerProProSerValAlaSerSerSerAlaSerGlrCGGCCGGGCGAAGGTTGGCGAGGGGATCACCCCCTCTGTGGCCAGCTCCTCGGCTAGCCGCCGGCCCGCTTCCAACCGCTCCCTAGTGGGGGGAGACACCGGTCGAGGAGCCGATCGG
4621	ProAspValAlaValLeuThrSerMetLeuThrAspProSerHisIleThrAlaGluAla AACCGGACGTGGCCGTGTTGACGTCCATGCTCACTGATCCCTCCC
4561	SerPheArgValGlyLeuHisGluTyrProValGlySerGlnLeuProCysGluProGluTATCATTCAGAGTAGGACTCCACGAATACCCGGTAGGGTCGCAATTACCTTGCGAGCCCGATAGTAAGTA
4501	AspGlyValargLeuHisArgPheAlaProProCysLysProLeuLeuArgGluGluValTGGACGGGGTGCGCCTACATAGGTTTGCGCCCCCTGCAAGCCCTTGCTGCGGGAGGAGGACCTTGCCCCACGCGGATGTATCCAAACGCGGGGGGACGTTCGGGAACGACGCCCTCCTCC
4441	thm:thm:thm:thm:thm:thm:thm:thm:thm:thm:
4381	SerAlaGluGluTyrValGluIleArgGlnValGlyAspPheHisTyrValThrGlyMet TGTCTGCAGAGGAATATGTGGAGATAAGGCAGGTGGGGGACTTCCACTACGTGACGGGTA ACAGACGTCTCCTTATACACCTCTATTCCGTCCACCCCCTGAAGGTGATGCACTGCCCAT
4321	ThrThrGlyProCysThrProLeuProAlaProAsnTyrThrPheAlaLeuTrpArgVal ACACCACGGGCCCCTGTACCCCCCTTCCTGCGCCGAACTACACGTTCGCGCTATGGAGGG TGTGGTGCCCGGGGACATGGGGGGAAGGACGCGGCTTGATGTGCAAGCGCGATACCTCCC
4261	IleValGlyProArgThrCysArgAsnMetTrpSerGlyThrPheProIleAsnAlaTyr GGATCGTCGGTCCTAGGACCTGCAGGAACATGTGGAGTGGGACCTTCCCCATTAATGCCT CCTAGCAGCCAGGATCCTGGACGTCCTTGTACACCTCACCCTGGAAGGGGTAATTACGGA
4201	HisThrArgCysHisCysGlyAlaGlulleThrGlyHisValLysAsnGlyThrMetArg TGCACACTCGCTGCCACTGTGGAGCTGAGATCACTGGACATGTCAAAAACGGGACGATGA ACGTGTGAGCGACGGTGACACCTCGACTCTAGTGACCTGTACAGTTTTTTGCCCTGCTACT
4141	IleProPheValSerCysGlnArgGlyTyrLysGlyValTrpArgValAspGlyIleMet GGATCCCCTTTGTGTCCTGCCAGCGCGGGTATAAGGGGGTCTGGCGAGTGGACGGCATCA CCTAGGGGAAACACAGGACGGTCGCGCCCATATTCCCCCAGACCGCTCACCTGCCGTAGT
4081	GluValleuSerAspPheLysThrTrpLeuLysAlaLysLeuMetProGlnLeuProGly GCGAGGTGTTGAGCGACTTTAAGACCTGGCTAAAAGCTAAGCTCATGCCACAGCTGCCTG CGCTCCACAACTCGCTGAAATTCTGGACCGATTTTCGATTCGAGTACGGTGTCGACGGAC
4021	SerGluCysThrThrProCysSerGlySerTrpLeuArgAsplleTrpAspTrplleCys GCTCGGAGTGTACCACTCCATGCTCCGGTTCCTGGCTAAGGGACATCTGGGACTGGATAT CGAGCCTCACATGGTGAGGTACGAGGCCCAAGGACCGATTCCCTGTAGACCCTGACCTATA
3961	AlaileLeuSerSerLeuThrValThrGlnLeuLeuArgArgLeuHisGlnTrpileSer CTGCCATACTCAGCAGCCTCACTGTAACCCAGCTCCTGAGGCGACTGCACCAGTGGATAA GACGGTATGAGTCGTCGGAGTGACATTGGGTCGAGGACTCCGCTGACGTGGTCACCTATT
3901	36/63 GGGGGAACCATGTTTCCCCCACGCACTACGTGCCGGAGAGCGATGCAGCTGCCCGCGTCA CCCCCTTGGTACAAAGGGGGTGCGTGATGCACGGCCTCTCGCTACGTCGACGGCCCAGT

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	3//03
4921	ArgGluIleSerValProAlaGluIleLeuArgLysSerArgArgPheAlaGlnAlaLeuAGCGGGAGATCTCGGTACCCGCAGAAATCCTGCGGAAGTCTCGGAGATTCGCCCAGGCCCTCGCCCTCTAGAGGCCATGGGCGTCTTTAGGACGCCTTCAGAGCCTCTAAGCGGGTCCGGG
4981	ProValTrpAlaArgProAspTyrAsnProProLeuValGluThrTrpLysLysProAspTGCCCGTTTGGGCGGCCGGCCGGACTATAACCCCCCGCTAGTGGAGACGTGGAAAAAGCCCGACCGGGCCAAACCCGCGCCCGGCCTGATATTGGGGGGGCGATCACCTCTGCACCTTTTTCGGGC
5041	TyrGluProProValValHisGlyCysProLeuProProProLysSerProProValProActAcGAAcCACCTGTGGTCCATGGCTGTCCGCTTCCACCTCCAAAGTCCCCTCCTGTGCTGATGCTTGGTGGACACCAGGTACCGACAGGCGAAGGTGGAGGTTTCAGGGGAGGACACG
5101	ProproArgLysLysArgThrValValLeuThrGluSerThrLeuSerThrAlaLeuAlaCTCCGCCTCGGAAGAAGCGGACGGTGGTCCTCACTGAATCAACCCTATCTACTGCCTTGGGAGGCGGAGCCTTCTTCGCCTGCCACCAGGAGTGACTTAGTTGGGATAGATGACGGAACC
5161	GluLeuAlaThrArgSerPheGlySerSerSerThrSerGlyIleThrGlyAspAsnThr CCGAGCTCGCCACCAGAAGCTTTGGCAGCTCCTCAACTTCCGGCATTACGGGCGACAATA GGCTCGAGCGGTGGTCTTCGAAACCGTCGAGGAGTTGAAGGCCGTAATGCCCGCTGTTAT
5221	ThrThrSerSerGluProAlaProSerGlyCysProProAspSerAspAlaGluSerTyrCGACAACATCCTCTGAGCCCGCCCTTCTGGCTGCCCCCCCGACTCCGACGCTGAGTCCTGCTGTTGTAGGAGACTCGGGCGGG
5281	SerSerMetProProLeuGluGlyGluProGlyAspProAspLeuSerAspGlySerTrpATTCCTCCATGCCCCCCTGGAGGGGAGCCTGGGGATCCTAGCGACCGAC
5341	SerThrValSerSerGluAlaAsnAlaGluAspValValCysCysSerMetSerTyrSerGGTCAACGGTCAGTAGTGAGGCCAACGCGGAGGATGTCGTGTGCTGCTCAATGTCTTACTCCAGTTGCCAGTCATCACTCCGGTTGCGCCTCCTACAGCACACGACGAGTTACAGAATGA
5401	TrpThrGlyAlaLeuValThrProCysAlaAlaGluGluGlnLysLeuProIleAsnAlaCTTGGACAGGCGCACTCGTCACCCCGTGCGCCGCGGAAGAACAGAAACTGCCCATCAATGGAACCTGTCCGCGTGAGCAGTGGGGCACGCGGCGCCCTTCTTGTCTTTGACGGGTAGTTAC
5461	LeuSerAsnSerLeuLeuArgHisHisAsnLeuValTyrSerThrThrSerArgSerAla CACTAAGCAACTCGTTGCTACGTCACCACAATTTGGTGTATTCCACCACCTCACGCAGTG GTGATTCGTTGAGCAACGATGCAGTGGTGTTAAACCACATAAGGTGGTGGAGTGCGTCAC
5521	CysGlnArgGlnLysLysValThrPheAspArgLeuGlnValLeuAspSerHisTyrGli CTTGCCAAAGGCAGAAGAAAGTCACATTTGACAGACTGCAAGTTCTGGACAGCCATTACC GAACGGTTTCCGTCTTCTTTCAGTGTAAACTGTCTGACGTTCAAGACCTGTCGGTAATGG
5581	AspValLeuLysGluValLysAlaAlaAlaSerLysValLysAlaAsnLeuLeuSerValAGGACGTACTCAAGGAGGTTAAAGCAGCGGCGTCAAAAGTGAAGGCTAACTTGCTATCCGTCCTGCATGAAGGTTCACTTCCGATTGAACGATAGGC
5641	GluGluAlaCysSerLeuThrProProHisSerAlaLysSerLysPheGlyTyrGlyAltTAGAGGAAGCTTGCAGCCTGACGCCCCCACACTCAGCCAAATCCAAGTTTGGTTATGGGGATCTCCTTCGAACGTCGGACTGCGGGGGTGTGAGTCGGTTTAGGTTCAAACCAATACCCC
5701	LysAspValArgCysHisAlaArgLysAlaValThrHisIleAsnSerValTrpLysAs CAAAAGACGTCCGTTGCCATGCCAGAAAGGCCGTAACCCACATCAACTCCGTGTGGAAAG GTTTTCTGCAGGCAACGGTACGGT
5761	LeuLeuGluAspAsnValThrProlleAspThrThrIleMetAlaLysAsnGluValPhaCCTTCTGGAAGACAATGTAACACCAATAGACACTACCATCATGGCTAAGAACGAGGTTTTGGAAGACCTTCTGTTACATTGTGGTTATCTGTGATGGTAGCAGATCCTGTCCAAA
5821	CysValGlnProGluLysGlyGlyArgLysProAlaArgLeuIleValPheProAspLe TCTGCGTTCAGCCTGAGAAGGGGGGTCGTAAGCCAGCTCGTCTCATCGTGTTCCCCCGATC AGACGCAAGTCGGACTCTTCCCCCCAGCATTCGGTCGAGCAGAGTAGCACAAGGGGCTAG
	GlyValArgValCysGluLysMetAlaLeuTyrAspValValThrLysLeuProLeuAl

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FIG. 32-6

5881	TGGGCG'IGCGCGTGTGCGAAAA ACCCGCACGCGCACACGCTTTT	AGATGGCTTTGTACGACGTGGTTACAAAGCTCCCCTTGG TCTACCGAAACATGCTGCACCAATGTTTCGAGGGGAACC	
5941	$-\alpha\alpha\alpha$	lyPheGlnTyrSerProGlyGlnArgValGluPheLeuVa GATTCCAATACTCACCAGGACAGCGGGTTGAATTCCTCG CTAAGGTTATGAGTGGTCCTGTCGCCCAACTTAAGGAGC	al
6001	TGCAAGCGTGGAAGTCCAAGAA ACGTTCGCACCTTCAGGTTCTT	ysThrProMetGlyPheSerTyrAspThrArgCysPheAs AAACCCCAATGGGGTTCTCGTATGATACCCGCTGCTTTG TTTGGGGTTACCCCAAGAGCATACTATGGGCGACGAAAC	
6061	ACTCCACAGTCACTGAGAGCGA TGAGGTGTCAGTGACTCTCGCT	spileArgThrGluGluAlaIleTyrGlnCysCysAsple ACATCCGTACGGAGGAGGCAATCTACCAATGTTGTGACC TGTAGGCATGCCTCCTCCGTTAGATGGTTACAACACTGG	
6121	TCGACCCCAAGCCCGCGTGGC AGCTGGGGGTTCGGGCGCACCG	lalleLysSerLeuThrGluArgLeuTyrValGlyGlyP; CCATCAAGTCCCTCACCGAGAGGCTTTATGTTGGGGGCC GGTAGTTCAGGGAGTGGCTCTCCGAAATACAACCCCCGG	
6181	CTCTTACCAATTCAAGGGGGGA GAGAATGGTTAAGTTCCCCCC	luAsnCysGlyTyrArgArgCysArgAlaSerGlyVall& AGAACTGCGGCTATCGCAGGTGCCGCGCGAGCGGCGTAC TCTTGACGCCGATAGCGTCCACGGCGCGCTCGCCGCATG	
6241	TGACAACTAGCTGTGGTAACAC ACTGTTGATCGACACCATTGTC	hrleuthrCystyrileLysAlaArgAlaAlaCysArgAl CCCTCACTTGCTACATCAAGGCCCGGGCAGCCTGTCGAG GGGAGTGAACGATGTAGTTCCGGGCCCGTCGGACAGCTC	
6301		hrMetLeuValCysGlyAspAspLeuValValI1eCysGl CCATGCTCGTGTGGCGACGACTTAGTCGTTATCTGTG GGTACGAGCACACCGCTGCTGAATCAGCAATAGACAC	Lu
6361	AAAGCGCGGGGGTCCAGGAGGI TTTCGCGCCCCCAGGTCCTCC	spAlaAlaSerLeuArgAlaPheThrGluAlaMetThrAi ACGCGGCGAGCCTGAGAGCCTTCACGGAGGCTATGACCA TGCGCCGCTCGGACTCTCGGAAGTGCCTCCGATACTGGT	
6421	<u>┍┍┉ᢆ</u> ᡑ┍ᡎᠬ᠘ᢗ᠘ᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗᢗ	spProProGlnProGluTyrAspLeuGluLeuIleThrSe ACCCCCACAACCAGAATACGACTTGGAGCTCATAACAT TGGGGGGTGTTGGTCTTATGCTGAACCTCGAGTATTGTA	≥r
6481	$-c\lambda mCCmCCmCC\lambda \lambda CCTCTC\lambda G$	alAlaHisAspGlyAlaGlyLysArgValTyrTyrLeuTi TCGCCCACGACGCGCTGGAAAGAGGGTCTACTACCTCA AGCGGGTGCTGCCGCGACCTTTCTCCCAGATGATGGAGT	ır
6541	CCCCTCACCCTACAACCCCCCC	euAlaArgAlaAlaTrpGluThrAlaArgHisThrProVa TCGCGAGAGCTGCGTGGGAGACAGCAAGACACACTCCAG AGCGCTCTCGACGCACCCTCTGTCGTTCTGTGAGGTC	al
6601	・ 中でも 5 中中ででではここで2 なこころ 2 ごと り	lelleMetPheAlaProThrLeuTrpAlaArgMetlleL TAATCATGTTTGCCCCCACACTGTGGGCGAGGATGATAC ATTAGTACAAACGGGGGTGTGACACCCGCTCCTACTATG	eu
6661	中になずになぐぐくなずずずでずずずれる(こ)(*	alleuIleAlaArgAspGlnLeuGluGlnAlaLeuAspC TCCTTATAGCCAGGGACCAGCTTGAACAGGCCCTCGATT AGGAATATCGGTCCCTGGTCGAACTTGTCCGGGAGCTAA	
6721	CCCACAMONACGGGGCCTGCT	YrSerileGluProLeuAspLeuProProIleIleGlnA ACTCCATAGAACCACTTGATCTACCTCCAATCATTCAAA TGAGGTATCTTGGTGAACTAGATGGAGGTTAGTAAGTTT	
6781	Leu GACTC CTGAG	FIG. 32-7	

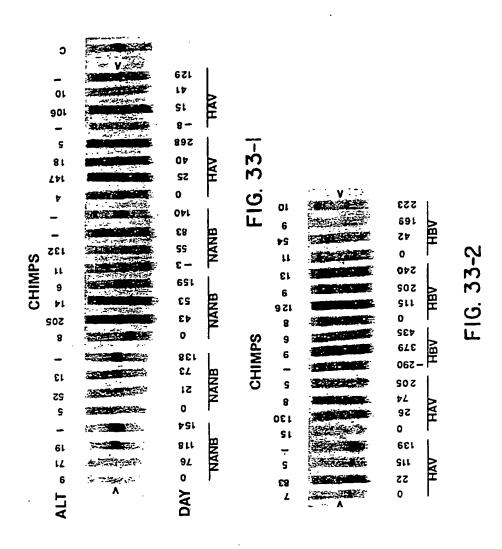
CHRSTITUTE SHEET

FIG. 33 LEGEND

1	Lane Number	Chimp Reference Number	Infection Type	Sample date (days) (0=inoculation day)	ALT (alanine) aminotransferase level in sera) mu/ml)
1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1			NAND	0	9
1 NANB 118					
1 NAME 154 N/A 5 2 NAMB 0 0 5 6 2 NAMB 21 52 7 2 NAMB 71 13 8 2 NAMB 138 N/A 9 3 NAMB 0 0 8 10 13 NAMB 0 0 8 10 10 3 NAMB 41 205 11 3 NAMB 53 14 12 3 NAMB 553 14 12 3 NAMB 553 14 12 13 NAMB 554 132 13 4 NAMB 555 132 14 4 NAMB 555 132 15 4 NAMB 83 1 N/A 15 4 NAMB 140 N/A 17 5 HAV 0 4 4 18 5 HAV 25 147 19 5 HAV 268 5 21 6 HAV 268 5 21 6 HAV 268 5 21 6 HAV 15 106 22 6 HAV 15 106 23 6 HAV 15 106 24 6 HAV 15 106 24 6 HAV 15 106 24 6 HAV 15 5 106 25 6 7 HAV 0 7 27 7 HAV 129 N/A 26 7 HAV 139 N/A 27 7 HAV 139 N/A 28 7 HAV 139 N/A 29 7 HAV 139 N/A 30 8 HAV 20 15 5 29 7 HAV 139 N/A 30 8 HAV 20 15 5 31 8 HAV 20 15 5 32 8 HAV 20 15 5 33 8 HAV 20 15 5 34 9 HBV 20 15 5 35 9 HBV 379 9 36 9 HBV 379 9 37 10 HBV 379 9 38 10 HBV 379 9 39 10 HBV 379 9 39 10 HBV 379 9 39 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 240 13				118	
5 2 NANB 21 52 6 2 NANB 21 52 7 2 NANB 73 13 8 2 NANB 13B N/A 9 3 NANB 0 0 8 10 3 NANB 41 205 11 3 NANB 51 14 12 3 NANB 51 159 6 11 4 NANB 51 159 6 11 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1				154	N/A
1	•			-	e
7 2 NANB 73 13 8 2 NANB 138 N/A 9 3 NANB 0 0 8 10 3 NANB 43 205 11 3 NANB 53 14 12 3 NANB 55 14 12 3 NANB 55 12 13 4 NANB 55 12 14 NANB 55 12 15 4 NANB 140 N/A 16 4 NANB 140 N/A 17 5 HAV 0 14 18 5 HAV 25 147 19 5 HAV 40 18 20 5 HAV 268 5 21 6 HAV 15 106 22 6 HAV 15 106 23 6 HAV 129 N/A 24 6 HAV 15 106 24 6 HAV 15 5 106 27 7 HAV 129 N/A 26 7 HAV 129 N/A 26 7 HAV 129 N/A 27 7 HAV 139 N/A 26 7 HAV 15 5 106 27 7 HAV 15 5 106 28 7 HAV 15 5 106 29 7 HAV 15 5 106 21 8 HAV 129 N/A 26 7 HAV 15 5 106 27 7 HAV 199 N/A 28 7 HAV 15 5 5 29 7 HAV 115 5 5 29 7 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 274 8 33 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV 290 N/A 35 9 HBV 379 9 36 9 HBV 1379 9 36 9 HBV 1379 9 37 10 HBV 270 N/A 38 10 HBV 270 N/A 39 10 HBV 240 13					
		2			
9				<u>-</u>	
10	8	2	NANB	130	1.7.1
10 3 NANB 43 205 11 3 NANB 53 14 12 3 NANB 53 14 14 NANB 53 159 6 13 4 NANB 55 132 15 4 NANB 55 132 15 4 NANB 83 N/A 16 5 HAV 0 40 18 20 5 HAV 25 147 19 5 HAV 40 18 20 5 HAV 268 5 21 6 HAV 268 5 21 6 HAV 15 106 22 6 HAV 15 106 23 6 HAV 15 106 24 6 HAV 15 5 106 27 7 HAV 129 N/A 26 7 HAV 129 N/A 27 7 HAV 129 N/A 26 7 HAV 139 N/A 27 7 HAV 139 N/A 28 7 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 26 110 32 8 HAV 26 110 33 8 HAV 26 110 34 9 HBV 26 110 35 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 9 37 10 HBV 379 9 38 10 HBV 379 9 39 10 HBV 379 9 36 9 HBV 379 9 37 10 HBV 379 9 38 10 HBV 205 5 37 10 HBV 379 9 38 10 HBV 379 9 39 10 HBV 379 9 36 9 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 240 13	9	3	NANB		=
11					
13 4 NANB -3 11 14 4 NANB 55 132 15 4 NANB 83 H/A 16 4 NANB 83 H/A 16 4 NANB 140 N/A 17 5 HAV 0 44 147 19 5 HAV 268 5 21 6 HAV 268 5 21 6 HAV 15 106 22 6 HAV 15 106 23 6 HAV 129 N/A 24 6 HAV 129 N/A 26 7 HAV 22 83 27 7 HAV 129 N/A 26 7 HAV 15 5 29 7 HAV 139 N/A 30 8 HAV 139 N/A 30 8 HAV 26 115 5 29 7 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 26 110 32 8 HAV 139 N/A 31 8 HAV 26 110 32 8 HAV 26 110 33 8 HAV 26 110 34 9 HBV 379 9 36 9 HBV 111-118 (pool) 96-156 (pool) 38 10 HBV 205 9 39 10 HBV 205 9 40 10 HBV 240 13		3	NANB		
113	12	3	NANB	159	6
113			NAME	_1	1.1
15 4 NANB 83 H/A 16 4 NANB 140 H/A 17 5 HAV 0 40 147 18 5 HAV 25 147 19 5 HAV 40 18 20 5 HAV 268 5 21 6 HAV 15 106 22 6 HAV 15 106 23 6 HAV 129 N/A 26 7 HAV 129 N/A 26 7 HAV 22 83 28 7 HAV 15 5 5 29 7 HAV 115 5 5 29 7 HAV 139 N/A 30 8 HAV 15 5 5 29 7 HAV 17 15 5 5 31 8 HAV 26 110 32 8 HAV 17 18 5 5 31 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV 379 9 35 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 9 37 10 HBV 205 9 38 10 HBV 379 6 37 10 HBV 205 9 38 10 HBV 205 9 40 10 HBV 205 9 40 10 HBV 205 13					
16 4 NANB 140 N/A 17 5 HAV 0 4 4 17 19 5 HAV 25 147 19 5 HAV 40 18 5 147 19 5 HAV 40 18 5 147 19 5 HAV 268 5 5 147 19 5 HAV 268 5 5 147 19 19 5 HAV 268 5 5 147 19 10 10 10 10 10 10 10 10 10 10 10 10 10		•			H/A
18 5 HAV 25 147 19 5 HAV 40 18 20 5 HAV 268 5 21 6 HAV -8 N/A 22 6 HAV 15 106 23 6 HAV 15 106 24 6 HAV 129 N/A 26 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 26 110 32 8 HAV 26 110 32 8 HAV 26 110 32 8 HAV 37 4 8 8 13 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 379 9 36 9 HBV 435 6 37 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13				140	и/Л
18 5 HAV 25 147 19 5 HAV 40 18 20 5 HAV 268 5 21 6 HAV -8 N/A 22 6 HAV 15 106 23 6 HAV 15 106 24 6 HAV 129 N/A 26 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 26 110 32 8 HAV 26 110 32 8 HAV 26 110 32 8 HAV 37 4 8 8 13 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 379 9 36 9 HBV 435 6 37 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13				_	
19 5 HAV 40 18 268 5 21 6 HAV -8 N/A 106 106 22 6 6 HAV 15 106 23 6 HAV 129 N/A 24 6 HAV 129 N/A 26 7 HAV 0 7 7 110 115 5 100 10 10 10 10 10 10 10 10 10 10 10 10		5			
20 5 HAV 268 5 21 6 HAV -8 N/A 22 6 HAV 15 106 23 6 HAV 41 10 24 6 HAV 129 N/A 26 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 139 N/A 30 8 HAV 26 130 31 8 HAV 26 130 32 8 HAV 74 8 33 8 HAV 70 8 33 8 HAV 70 9 36 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 6 37 10 HBV 379 6 38 10 HBV 379 6 39 10 HBV 379 6 39 10 HBV 205 9 40 10 HBV 240 11 41 11 HBV 240 11 41 11 HBV 240 11 41 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169		5			
21 6 HAV -8 N/A 22 6 HAV 15 106 23 6 HAV 41 10 24 6 HAV 129 N/A 26 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 139 N/A 30 8 HAV 26 110 32 8 HAV 274 8 33 8 HAV 205 5 34 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 379 6 37 10 HBV 379 6 38 10 HBV 379 6 39 10 HBV 205 9 40 10 HBV 240 13		5			
22 6 HAV 15 106 23 6 HAV 41 10 24 6 HAV 129 N/A 26 7 HAV 0 7 27 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 9 HAV 139 N/A 30 9 HAV 26 130 31 8 HAV 26 130 32 9 HAV 74 8 33 8 HAV 205 5 34 9 HBV 74 8 35 9 HBV 379 9 36 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 435 6 37 10 HBV 111-118 (pool) 96-156 (pool) 939 10 HBV 240 13 41 11 HBV 240 13	20	>	HAV	266	,
22 6 HAV 15 106 23 6 HAV 41 10 24 6 HAV 129 N/A 26 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 115 5 31 8 HAV 139 N/A 30 8 HAV 26 110 32 8 HAV 374 8 33 8 HAV 205 5 34 9 HBV 205 5 34 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 435 6 37 10 HBV 435 6 37 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 240 13 41 11 HBV 240 13 41 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169	21	. 6	HAV	-8	N/A
23 6 HAV 41 10 N/A 24 6 HAV 129 N/A 26 7 HAV 0 7 7 7 11 15 5 5 15 15 15 15 15 15 15 15 15 15				15	106
24 6 HAV 129 N/A 26 7 HAV 0 77 27 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 0 HAV 26 110 31 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV 379 36 9 HBV 379 36 9 HBV 379 36 9 HBV 435 37 10 HBV 435 38 10 HBV 205 37 10 HBV 205 38 10 HBV 205 39 10 HBV 205 40 10 HBV 240 11 HBV 26 41 11 HBV 26 42 11 HBV 240 11 11 HBV 240 11 10 (pool) 8-100 (pool) 410 41 11 HBV 240 43 10 HBV 240 43 11 HBV 26 100 (pool) 8-100 (pool) 410					
27 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV 379 36 9 HBV 379 36 9 HBV 435 37 10 HBV 435 37 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 40 10 HBV 240 13 41 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169	24	6	VAH	129	N/A
27 7 HAV 22 83 28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 26 110 31 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV 379 36 9 HBV 379 36 9 HBV 435 37 10 HBV 435 37 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 40 10 HBV 240 13 41 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169		_		•	7
28 7 HAV 115 5 29 7 HAV 139 N/A 30 8 HAV 0 155 31 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 435 6 37 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 112-118 (pool) 8-100 (pool) 43 11 HBV 169 199					
29 7 HAV 139 N/A 30 8 HAV 0 15 31 8 HAV 26 130 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 37 10 HBV 435 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 240 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169					
30 8 HAV 0 15 31 8 HAV 26 130 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 435 6 37 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 240 13 41 11 HBV 0 16901 8-100 (pool) 43 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 269 190					
31 8 HAV 26 110 32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 37 10 HBV 435 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 240 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169	- 2	•			
32 8 HAV 74 8 33 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 37 10 HBV 435 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 111-118 (pool) 96-156 (pool) 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169		8			
33 8 HAV 205 5 34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 0 8 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 111-118 (pool) 8-100 (pool) 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9					
34 9 HBV -290 N/A 35 9 HBV 379 9 36 9 HBV 435 6 37 10 HBV 0 8 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 111-118 (pool) 8-100 (pool) 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169					
34 9 HBV 379 9 9 379 9 9 36 9 HBV 435 6 6 9 HBV 435 6 6 9 HBV 435 6 6 9 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 13 13 11 HBV 240 13 13 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9 19	33	8	HAV	203	,
35 9 HBV 379 36 9 HBV 435 37 10 HBV 0 8 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169	3.4	0	нву	-290	
36 9 HBV 435 6 37 10 HBV 0 8 38 10 HBV 111-118 (pool) 96-156 (pool) 39 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169					
37 10			квv	435	. 6
37 10	- '			^	g
38 10 HBV 205 9 40 10 HBV 240 13 41 11 HBV 0 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9					
40 10 HBV 240 13 41 11 HBV 0 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9					
41 11 HBV 0 11 42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9			• • • •		
41 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9	40	10	1104	-	
42 11 HBV 28-56 (pool) 8-100 (pool) 43 11 HBV 169 9	41	11	иву		
43 11 10					
44 11 HBV 223					
	44	11	HRV	443	, ••

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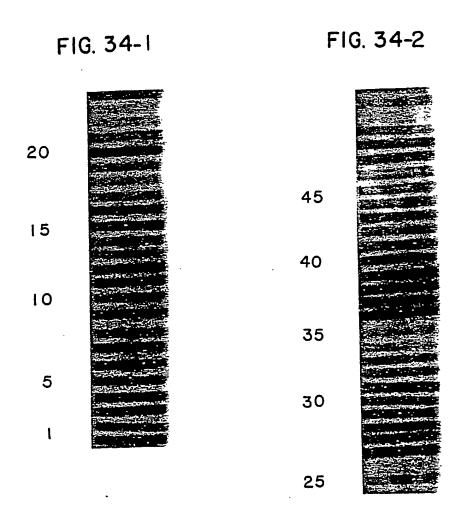


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FIG. 34 LEGEND

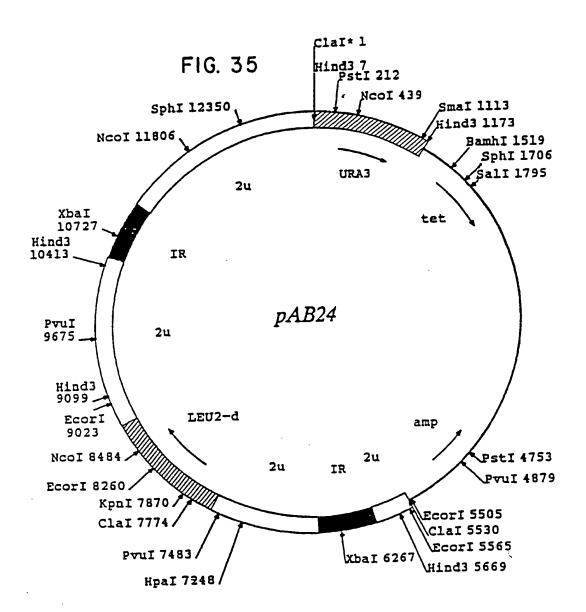
		110.01 220-	
	Patient		
Lane	Reference	Diagnosis	ALT Level (mu/ml)
Number	Number	Diagnosis	
	, 1	NANB	1354
1		NANB	31
2	1.1	NANB	14
3	21	NANB	79
4	11 21 21 21 31 31	NANB NANB	26
5	2 1		78
5 6	3;	NANB	87
7	3 ;	NANB	25
8	31	NANB	60
9	41	NANB	13
10	41	ВИДИ	298
11	51 51	Види	101
12	5 !	NANB	474
13	6 l	Види	318
14	61	NANB	20
15	71	Види	163
16	71	NANB	44
17	ģ1	NANB	
18	ğl	NANB	50
19	9	nanb	N/A
20	10	NANB	N/A
21	11	NANB	N/A
			N/A
22	12	Normal	N/A
23	13	Normal	и/А
24	14	Normal	N/A
26	30174	Normal	N/A
27	30105	Normal	N/A
	30103	Normal	N/A
28	30072	Normal	N/A
29	30146	Normal	N/A
30	30250	Normal	N/A
31	30230	Normal	N/A
32		AcuteHAV	N/A
33	15 16	AcuteHAV	N/A
34	17	AcuteHAV	N/A
35	18	AcuteHAV	N/A
36 37	48088	AcuteHAV	N/A
38	47288	AcuteHAV	N/A
39	47050	AcuteHAV	N/A
40	46997	AcuteHAV	N/A
	19	Convalescent HBV	N/A
41		(anti-HBSag+ve;	N/A
42	20		N/A
43	21	anti-HBCag+ve; (anti-HBSag+ve;	N/A
44	22		N/A
45	23	anti-HBCag+ve)	N/A
46	24	(anti-HBSag+ve;	N/A
47	25	anti-HBCag+ve)	N/A
48	26	(anti-HBSag+ve;	. N/A
49	27	anti-HBSag+ve)	**/ #1

 $^{^{1}}$ Sequential serum samples were assayed from these patients



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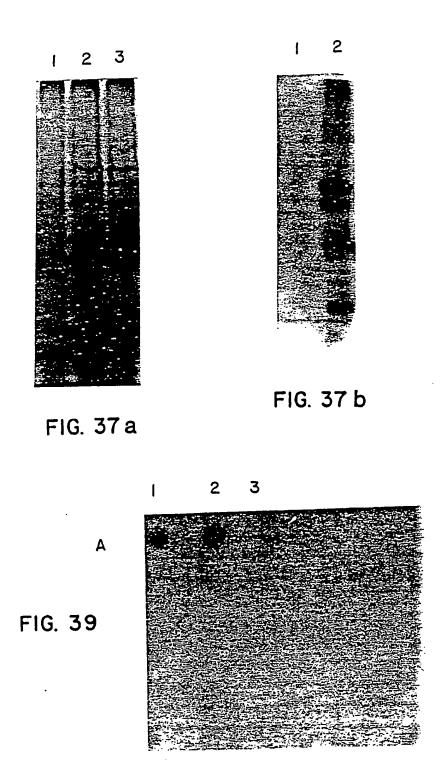
	7.11.00
FIG.	36-1 COOH-terminus of SOD-C100 Fusion Polypeptide
• -	NANBHpolypeptide
1	SODCOOH][adaptor][NANBHpolypeptide> AlaCysGlyVallleGlyIleAlaGlnAsnLeuGlyIleArgAspAlaHisPheLeuSer GCTTGTGGTGTAATTGGGATCGCCCAGAATTTGGGAATTCGGGATGCCCACTTTCTATCC CGAACACCACATTAACCCTAGCGGGTCTTAAACCCTTAAGCCCTACGGGTGAAAGATAGG
	>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
61	GlnThrLysGlnSerGlyGluAsnLeuProTyTLeuValAtaTytGliAtaTit ValCyC CAGACAAAGCAGAGTGGGGAGAACCTTCCTTACCTGGTAGCGTACCAAGCCACCGTGTGC GTCTGTTTCGTCTCACCCCTCTTGGAAGGAATGGACCATCGCATGGTTCGGTGGCACACG
121	AlaArgAlaGlnAlaProProProSerTrpAspGlnMetTrpLysCysLeulleArgLeu GCTAGGGCTCAAGCCCCTCCCCCATCGTGGGACCAGATGTGGAAGTGTTTGATTCGCCTC CGATCCCGAGTTCGGGGAGGGGGTAGCACCCTGGTCTACACCTTCACAAACTAAGCGGAG
	LysProThrLeuHisGlyProThrProLeuLeuTyrArgLeuGlyAlaValGlnAsnGlu
181	AAGCCCACCCTCCATGGGCCAACACCCCTGCTATACAGACTGGGCGGTATACAGATCTTACTT TTCGGGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGACCCGCGACAAGTCTTACTT
	IleThrLeuThrHisProValThrLysTyrIleMetThrCysMetSerAlaAspLeuGlu
241	ATCACCCTGACGCACCCAGTCACCAAATACATCATGACATGCATG
	ValValThrSerThrTrpValLeuValGlyGlyValLeuAlaAlaLeuAlaAlaTyrCys
301	GTCGTCACGAGCACCTGGGTGCTTCGTTGGCGGCGTCCTGGCTGCTTTGGCCGCGTATAACG CAGCAGTGCTCGTGGACCCACGAGCAACCGCCGCAGGACCGACGAAACCGGCGCATAACG
361	LeuSerThrGlyCysValVallleValGlyArgValValLeuSerGlyLysProAlaIle CTGTCAACAGGCTGCGTGGTCATAGTGGGCAGGGTCGTCTTGTCCGGGAAGCCGGCAATC GACAGTTGTCCGACGCACCAGTATCACCCGTCCCAGCAGAACAGGCCCTTCGGCCGTTAG
421	IleProAspArgGluValLeuTyrArgGluPheAspGluMetGluGluCysSerGlnHis ATACCTGACAGGGAAGTCCTCTACCGAGAGTTCGATGAGATGGAAGAGTGCTCTCAGCAC TATGGACTGTCCCTTCAGGAGATGGCTCTCAAGCTACTCTACCTTCTCACGAGAGTCGTG
481	LeuProTyrlleGluGlnGlyMetMetLeuAlaGluGlnPheLysGlnLysAlaLeuGly TTACCGTACATCGAGCAAGGGATGATGCTCGCCGAGCAGTTCAAGCAGAAGGCCCTCGGC AATGGCATGTAGCTCGTTCCCTACTACGAGCGGCTCGTCAAGTTCGTCTTCCGGGAGCCG
541	GAGGACGTCTGGCGCAGGCAGTCCGTCTCCAATAGCGGGGACGACAGGTCTGGTTGACC
601	GTTTTTGAGCTCTGGAAGACCCGCTTCGTATACACCTTGAAGTAGTCACCCTATGTTATG
661	LeualaglyLeuSerthrLeuProglyAsnProalallealaSerLeuMetalaPheThr TTGGCGGGCTTGTCAACGCTGCCTGGTAACCCCGCCATTGCTTCATTGATGGCTTTTACA AACCGCCCGAACAGTTGCGACGGACCATTGGGGCGGTAACGAAGTAACTACCGAAAATGT
721	CGACGACAGTGGTCGGGTGATTGGTGATCGGTTTGGGAGGAGGTTGTATAACCCCCCC
781	TrpValAlaAlaGlnLeuAlaAlaProGlyAlaAlaThrAlaPheValGlyAlaGlyLeu TGGGTGGCTGCCCAGCTCGCCGCCCCCGGTGCCGCTACTGCCTTTGTGGGCGCTGGCTTA ACCCACCGACGGGTCGAGCGGCGGGGGCCACGGCGATGACGGAAACACCCGCGACCGAAT

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AlaGlyAlaAlaIleGlySerValGlyLeuGlyLysValLeuIleAspIleLeuAlaGly
841 GCTGGCGCCGCCATCGGCAGTGTTGGACTGGGGAAGGTCCTCATAGACATCCTTGCAGGG

	CGACCGCGGCGGTAGCCGTCACAACCTGACCCCTTCCAGGAGTATCTGTAGGAACGTCCC
901	TyrGlyAlaGlyValAlaGlyAlaLeuValAlaPheLysIleMetSerGlyGluValPro TATGGCGCGGGCGTGGCGGAGCTCTTGTGGCATTCAAGATCATGAGCGGTGAGGTCCCC ATACCGCGCCCCGCACCGCCCTCGAGAACACCGTAAGTTCTAGTACTCGCCACTCCAGGGG
961	SerThrGluAspLeuValAsnLeuLeuProAlaIleLeuSerProGlyAlaLeuValVal TCCACGGAGGACCTGGTCAATCTACTGCCCGCCATCCTCTCGCCCGGAGCCCTCGTAGTC AGGTGCCTCCTGGACCAGTTAGATGACGGGCGGTAGGAGAGCGGGCCTCGGGAGCATCAG
L021	GlyValValCysAlaAlaIleLeuArgArgHisValGlyProGlyGluGlyAlaValGln GGCGTGGTCTGTGCAGCAATACTGCGCCGGCACGTTGGCCCGGGCGAGGGGGCAGTGCAG CCGCACCAGACACGTCGTTATGACGCGGCCCGTGCAACCGGGCCCGCTCCCCCGTCACGTC
L081	CCCCCCCTTGGTACAAAGGGGTCAGGTAGTAGTAGTAGTAGTAGTAGTAGTAGTAGTAGTAGT
1141] Lysargop AAGCGTTGACGCTCCCTACGGGTGGACTGTGGAGAGACAGGGCACTGCTAAGGCCCAAAT TTCGCAACTGCGAGGGATGCCCACCTGACACCTCTCTGTCCCGTGACGATTCCGGGTTTA
1201	CTCAGCCATGCATCGAGGGGTACAATCCGTATGGCCAACAACTAGCGCGTACGTA
1261	TCCTTTCTCGATGGTCCATACCTTAGATGCGTTAGCATTAATCCGAATTC AGGAAAGAGCTACCAGGTATGGAATCTACGCAATCGTAATTAGGCTTAAG

FIG. 36-2



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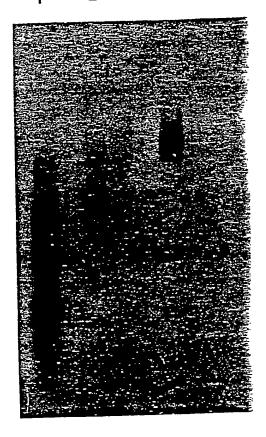
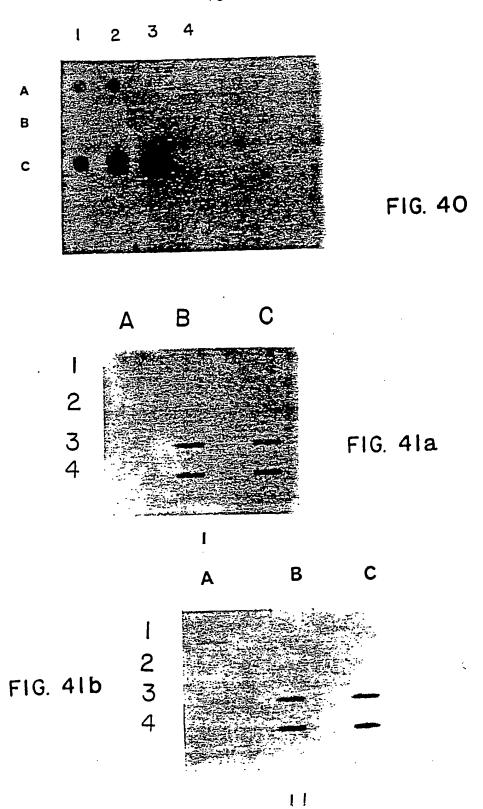


FIG. 38



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FIG. 41-1 Homology between the HCV polypeptide encoded by combined ORF of clones 14i through 39c) and the non-structural protein of the Dengue flavivirus(MNWVDl). EYVVLLFLLLADARVCSCLWMMLLISQAEAALENLVILNAASLAGTHGLVSFLVFFCFA HCV AVSFVTLITGNMSFRDLGRVMVMVGATMTDDIGMGVTYLALLAAFKVRPTFAAGLLLRKL MNWVDl в0 WYLKGKWYPGAVYTFYGMWPLLLLLLALPORAYALDTEVAASCGGVVLVGLMALTLSPYY **HCV** TSKELMMTTIGIVLLSQSTIPETILELTDALALGMMVLKMVRKMEKYQLAVTIMAILCVP MNWVDl KRYISWCLWWLQYFLTRVEAQLHVWIPPLNVRGGRDAVILLMCAVHPTLVFDITKLLLAV **HCV** NAVILONAWKVSCTILAVVSVSPLFLTSSQQKADWIPLALTIKGLNPTAIF-LTTLSRTN MNWVDl FGPLWILQASILKVPYF-VRVQGLLRF-CALARKMIGGHYVQMVIIKLGALTGTYVYNHL **HCV** . . .::. KKRSWPLNEAIMAVGMVSILASSLLKNDIPMTGPLVAGGILTVCYV-LTGRSADLELERA MNWVDl TPLRDWAHNGLRDLAVAVEPVVFSQMETKLITWGADTAACGDIINGLPVSARRGREILLG **HCV** . : ADVK-WEDQAEISGSSPILSITISE-DGSMSIKNEEEEQTLTILIRTGLLVISG---LFP MNWVDl · 410 PADGMVSKGWRLLAPITAYAQQTRGLLGCIITSLTGRDKNQVEGEVQIVSTAAQTFLATC HCV VSIPITAAAWYLWEVKKORAGVLWDVPSPPPVGKAELEDGAYRIKOKGILGYSQIGAGVY MNWVDl INGVCWTVYHGAGTRTIASPKGPVIQMYTNVDQDLV---GWPAPQGSRSLTPCTCGSSD HCV KEGTFHTMWHVTRGAVLMHKGKRIEPSWADVKKDLVSCGGGWKLEGEWKEGEEVQVLALE MNWVDl LYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGIFRAAVCTRGV **HCV** :.::... 1..... PGKNPRAVOTKPGLFKTN--AGTIGAVSLDFSPGTSGSPIIDKKGKVVGLYGNGVVTRSG MNWVDl AKAVDFIPVENLETTMRSPVFTDNSSPPVVPQSFQVAHLHAPTGSGKS--TKVPAAYAAQ HCV AYVSAIAQTEK--SIEDNPEIEDDIFRK---RKLTIMDLHPGAGKTKRYLPAIVRGAIKR MNWVDl

HCV MNWVD1	GYKVLVLNPSVAATLGFGAYMSKAHGIDPNIRTGVRTITTGSPITYSTYGKFLADGGC GLRTLILAPTRVVAAFMEEALRGLPIRYOTPAIRAEHTGREIVDLMCHATFTMRIL-SPV 650 660 670 680 690 700
MNWVD1	590 600 610 620 630 640 SGGAYDIIICDECHSTDATSILGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEEV . X::::::::::::::::::::::::::::::::::
HCV MNWVDl	650 660 670 680 690 700 ALSTTGEIPFYGKAIPLEVIKGGRHLIFCHSKKKCDELAAKLVALGINAVAYYRGLDVSV:: IMDEEREIPERSWSSGHEWVTDFKGKTVWFVPSIKAGNDTAACLRKNGKKVTQLSRKTFD 770 780 790 800 810 820
HCV MNWVD1	710 720 730 740 750 760 IPTSGDVVVVATDALMTGYTGDFDSVIDCNTCVTQTVDFSLDPTFTIETITLPQDAVSRT SEYVKTRINDWNFVVTTDISEMGANFKAERVIDPRRCMKPVILTDGEERVILAGPMPVTH 830 840 850 860 870 880
HCV MNWVDl	770 780 790 800 810 820 QRRGRTGRGKPGIYRFVAPGERPSGMFDSSVLCECYDAGCAWYELTPAETTVRLRAYMNT SS

FIG. 41-2

FIG. 43
DISTRIBUTION OF RANDOM SAMPLES

C100-3 Ag ELISA Preclinical Kit 416ng C100/WELL, 2 HRS 37°C, 20ul SAMPLE

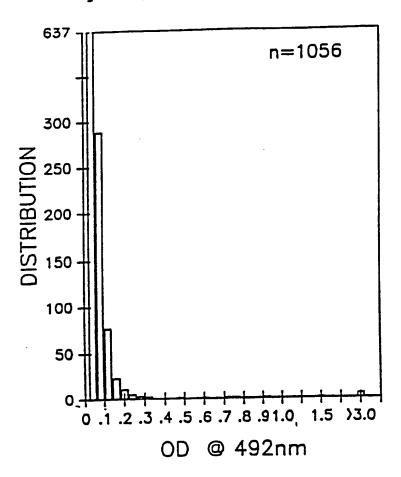


FIG. 44

Distribution of O.D. Values for

Random Blood Donor Samples Tested with Two ELISA

Configurations

C100-3 Ag ELISA MoAB vs Polyclonai

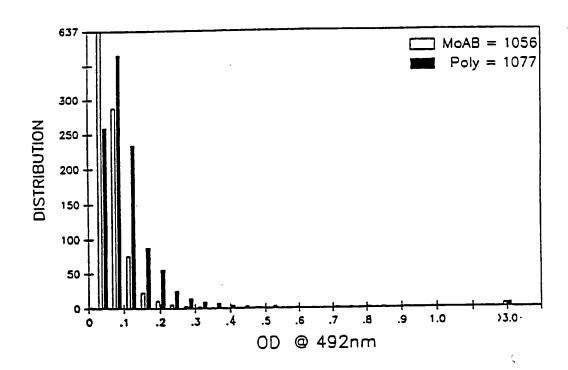


FIG. 45

	110. 75	_	_
Name	Common Sequence	Variable	Sequence
		ССУД	CTTGC
5'-3-1	AAGCTTGATCGAATTC		CCTGC
-2			CATGC
-3			CGTGC
-4			
- 5			GTTGC
-6		CGAA	GCTGC
-7			CTTGC
-8			CCTGC
-9			CATGC
-10		AGAT	CGTGC
-10 -11			GTTGC.
-11 -12		AGAA	GCTGC
		CGAT	CTTGT
-13		CGAT	CCTGT
-14	• •		CATGT
-15			CGTGT
-16			GTTGT
-17		CCA	GCTGT
-18		-	
-19		AGAT	CTTGT
-19 -20		AGAT	CCTGT
		AGAT	CATGT
-21	•		CGTGT
-22			GTTGT
-23			AGCTGT
-24			
-25			CTTGC
-26 -26	•		CCTGC
-27			CATGC
-28			CGTGC
-29		CGC	AGTTGC
-30			AGCTGC
-30	•		
-31	•		CTTGT
-32	•		rcctgt `
-33			CATGT
-34			rcgtgt
-35			AGTTGT
-36		CGC	AGCTGT
-20			

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1	F	IG	46	- I	Translation	of	DNA	k9-1
ı	_	IU.	70		17011010-			

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- GlyCysProGluArgLeuAlaSerCysArgProLeuThrAspPheAspGlnGlyTrpGly
 CAGGCTGTCCTGAGAGGCTAGCCAGCTGCCGACCCCTTACCGATTTTGACCAGGGCTGGG
 GTCCGACAGGACTCTCCGATCGGTCGACGGCTGGGGAATGGCTAAAACTGGTCCCGACCC
- ProlleSerTyrAlaAsnGlySerGlyProAspGlnArgProTyrCysTrpHisTyrPro 61 GCCCTATCAGTTATGCCAACGGAAGCGGCCCCGACCAGCGCCCCTACTGCTGGCACTACC CGGGATAGTCAATACGGTTGCCTTCGCCGGGGGTCGCGGGGATGACGACCGTGATGG
- ProSerProValValValGlyThrThrAspArgSerGlyAlaProThrTyrSerTrpGly
 181 CTCCCAGCCCGTGGTGGTGGGAACGACCGACAGGTCGGGCGCGCCCCACCTACAGCTGGG
 GAGGGTCGGGGCACCACCACCCTTGCTGCTGCTCCAGCCCGCGGGTGGATGTCGACCC
- GluAsnAspThrAspValPheValLeuAsnAsnThrArgProProLeuGlyAsnTrpPhe
 241 GTGAAAATGATACGGACGTCTTCGTCCTTAACAATACCAGGCCACCGCTGGGCAATTGGT
 CACTTTTACTATGCCTGCAGAAGCAGGAATTGTTATGGTCCGGTGGCGACCCGTTAACCA
- GlyCysThrTrpMetAsnSerThrGlyPheThrLysValCysGlyAlaProProCysVal
 TCGGTTGTACCTGGATGAACTCAACTGGATTCACCAAAGTGTGCGGAGCGCCTCCTTGTG
 AGCCAACATGGACCTACTTGAGTTGACCTAAGTGGTTTCACACGCCTCGCGGAGGAACAC
- IleGlyGlyAlaGlyAsnAsnThrLeuHisCysProThrAspCysPheArgLysHisPro
 361 TCATCGGAGGGGCGACAACACCCTGCACTGCCCCACTGATTGCTTCCGCAAGCATC
 AGTAGCCTCCCCGCCCGTTGTTGTGGGACGTGACGGGGTGACTAACGAAGGCGTTCGTAG
- AspalaThrTyrSerArgCysGlySerGlyProTrplleThrProArgCysLeuValAsp
 421 CGGACGCCACATACTCTCGGTGCGGCTCCCGGTCCCTGGATCACACCCAGGTGCCTGGTCG
 GCCTGCGGTGTATGAGAGCCACGCCGAGGCCAGGGACCTAGTGTGGGTCCACGGACCAGC
- TyrProTyrArgLeuTrpHisTyrProCysThrlleAsnTyrThrllePheLyslleArg
 481 ACTACCCGTATAGGCTTTGGCATTATCCTTGTACCATCAACTACACTATATTTAAAATCA
 TGATGGCCATATCCGAAACCGTAATAGGAACATGGTAGTTGATGTGATATAAATTTTAGT
- ArgCysAspLeuGluAspArgAspArgSerGluLeuSerProLeuLeuThrThr
 601 AACGTTGCGATCTGGAAGATAGGGACAGGTCCGAGCTCAGCCCGTTACTGCTGACCACTA
 TTGCAACGCTAGACCTTCTATCCCTGTCCAGGCTCGAGTCGGGCAATGACGACTGGTGAT
- GlnTrpGlnValleuProCysSerPheThrThrLeuProAlaLeuSerThrGlyLeuIle
 661 CACAGTGGCAGGTCCTCCCGTGTTCCTTCACAACCCTGCCAGCCTTGTCCACCGGCCTCA
 GTGTCACCGTCCAGGAGGGCACAAGGAAGTGTTGGGACGGTCGGAACAGGTGGCCGGAGT
- ----Overlap with Combined ORF of DNAs 12f through 15e----HisLeuHisGlnAsnIleValAspValGlnTyrLeuTyrGlyValGlySerSerIleAla
 TCCACCTCCACCAGAACATTGTGGACGTGCAGTACTTGTACGGGGTGGGGTCAAGCATCG
 AGGTGGAGGTGGTCTTGTAACACCTGCACGTCATGAACATGCCCCACCCCAGTTCGTAGC
- SerTrpAlaIleLysTrpGluTyrValValLeuLeuPheLeuLeuLeuAlaAspAlaArg
 781 CGTCCTGGGCCATTAAGTGGGAGTACGTCGTCCTCTGTTCCTTCTGCTTGCAGACGCGC
 GCAGGACCCGGTAATTCACCCTCATGCAGCAGGACGACAAGGAAGACGAACGTCTGCGCG

841	ValCysSerCysLeuTrpMetMetLeuLeuIleSerGlnAlaGluAlaAlaLeuGluAsn GCGTCTGCTCCTGCTTGTGGATGATGCTACTCATATCCCAAGCGGAAGCGGCTTTGGAGA CGCAGACGAGGACGAACACCTACTACGATGAGTATAGGGTTCGCCTTCGCCGAAACCTCT
901	LeuValIleLeuAsnAlaAlaSerLeuAlaGlyThrHisGlyLeuValSerPheLeuVal ACCTCGTAATACTTAATGCAGCATCCCTGGCCGGGACGCACGGTCTTGTATCCTTCCT
961	PhePheCysPheAlaTrpTyrLeuLysGlyLysTrpValProGlyAlaValTyrThrPheTGTTCTTCTGCTTTGCATGGTATCTGAAGGGTAAGTGGGTGCCCGGAGCGGTCTACACCTACAAGAAGAAGACGAAACGTACCATAGACTTCCCATTCACCCCACGGGCCTCGCCAGATGTGGA
1031	TyrGlyMetTrpProLeuLeuLeuLeuLeuleuAlaLeuProGlnArgAlaTyrAlaLeu TCTACGGGATGTGGCCTCCTCCTCCTCCTGCTCCTGTTGCCCCAGCGGCGGCGTACGCGC AGATGCCCTACACCGGAGAGGAGGACGAGGACAACCGCAACGGGGTCGCCCGCATGCGCG
1081	AspThrGluValAlaAlaSerCysGlyGlyValValLeuValGlyLeuMetAlaLeuThr TGGACACGGAGGTGGCCGCGTCGTGTGGCGGTTGTTCTCGTCGGGTTGATGGCGCTAA ACCTGTGCCTCCACCGGCGCAGCACCACCACCAACAAGAGCAGCCCAACTACCGCGATT
1141	LeuSerProtyrtyrLysArgTyrlleSerTrpCysLeuTrpTrpLeuGlnTyrPheLeu CTCTGTCACCATATTACAAGCGCTATATCAGCTGGTGCTTGTGGTGGCTCAGTATTTTC GAGACAGTGGTATAATGTTCGCGATATAGTCGACCACGAACACCACCGAAGTCATAAAAG
1201	ThrargValGluAlaGlnLeuHisValTrpIleProProLeuAsnValArgGlyGlyArg TGACCAGAGTGGAAGCGCAACTGCACGTGTGGATTCCCCCCCTCAACGTCCGAGGGGGGC ACTGGTCTCACCTTCGCGTTGACGTGCACACCTAAGGGGGGGAGTTGCAGGCTCCCCCG
1261	AspAlaValIleLeuLeuMetCysAlaValHisProThrLeuValPheAspIleThrLys GCGACGCTGTCATCTTACTCATGTGTGCTGTACACCCGACTCTGGTATTTGACATCACCA CGCTGCGACAGTAGAATGAGTACACACGACATGTGGGCTGAGACCATAAACTGTAGTGGT
1321	LeuLeuAlaValPheGlyProLeuTrpIleLeuGlnAla AATTGCTGCTGGCCGTCTTCGGACCCCTTTGGATTCTTCAAGCCAG TTAACGACGACCGCCAGAAGCCTGGGGAAACCTAAGAAGTTCGGTC

FIG. 46-2

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FIG. 47-1 COMBINED ORF OF DNAs K9-1 through 15e

- GlyCysProGluArgLeuAlaSerCysArgProLeuThrAspPheAspGlnGlyTrpGly
 CAGGCTGTCCTGAGAGGCTAGCCAGCTGCCGACCCCTTACCGATTTTGACCAGGGCTGGG
 GTCCGACAGGACTCTCCGATCGGTCGACGGCTGGGGAATGGCTAAAACTGGTCCCGACCC
- ProlleSerTyrAlaAsnGlySerGlyProAspGlnArgProTyrCysTrpHisTyrPro 61 GCCCTATCAGTTATGCCAACGGAAGCGGCCCCGACCAGCGCCCCTACTGCTGGCACTACC CGGGATAGTCAATACGGTTGCCTTCGCCGGGGCTGGTCGCGGGGATGACGACCGTGATGG

- GluAsnAspThrAspValPheValLeuAsnAsnThrArgProProLeuGlyAsnTrpPhe 241 GTGAAATGATACGGACGTCTTCGTCCTTAACAATACCAGGCCACCGCTGGGCAATTGGT CACTTTTACTATGCCTGCAGAAGCAGGAATTGTTATGGTCCGGTGGCGACCCGTTAACCA
- GlyCysThrTrpMetAsnSerThrGlyPheThrLysValCysGlyAlaProProCysVal
 TCGGTTGTACCTGGATGAACTCAACTGGATTCACCAAAGTGTGCGGAGCGCCTCCTTGTG
 AGCCAACATGGACCTACTTGAGTTGACCTAAGTGGTTTCACACGCCTCGCGGAGGAACAC
- IleGlyGlyAlaGlyAsnAsnThrLeuHisCysProThrAspCysPheArgLysHisPro
 361 TCATCGGAGGGGGGCAACAACACCCTGCACTGCCCCACTGATTGCTTCCGCAAGCATC
 AGTAGCCTCCCCGCCCGTTGTTGTGGGACGTGACGGGGTGACTAACGAAGGCGTTCGTAG
- AspalaThrTyrSerArgCysGlySerGlyProTrpIleThrProArgCysLeuValAsp
 421 CGGACGCCACATACTCTCGGTGCGGCTCCCTGGATCACACCCAGGTGCCTGGTCG
 GCCTGCGGTGTATGAGAGCCACGCCGAGGCCAGGGACCTAGTGTGGGTCCACGGACCAGC
- TyrProTyrArgLeuTrpHisTyrProCysThrlleAsnTyrThrllePheLysIleArg
 481 ACTACCCGTATAGGCTTTGGCATTATCCTTGTACCATCAACTACACCATATTTAAAATCA
 TGATGGGCATATCCGAAACCGTAATAGGAACATGGTAGTTGATGTGGTATAAATTTTAGT
- ArgCysAspLeuGluAspArgAspArgSerGluLeuSerProLeuLeuLeuThrThrThr
 601 AACGTTGCGATCTGGAAGACAGGGACAGGTCCGAGCTCAGCCCGTTACTGCTGACCACTA
 TTGCAACGCTAGACCTTCTGTCCCTGTCCAGGCTCGAGTCGGGCAATGACGACTGGTGAT
- GlnTrpGlnValLeuProCysSerPheThrThrLeuProAlaLeuSerThrGlyLeuIle
 661 CACAGTGGCAGGTCCTCCCGTGTTCCTTCACAACCCTACCAGCCTTGTCCACCGGCCTÇA
 GTGTCACCGTCCAGGAGGGCACAAGGAAGTGTTGGGATGGTCGGAACAGGTGGCCGGAGT
- HisLeuHisGlnAsnIleValAspValGlnTyrLeuTyrGlyValGlySerSerIleAla
 721 TCCACCTCCACCAGAACATTGTGGACGTGCAGTACTTGTACGGGGTGGGGTCAAGCATCG
 AGGTGGAGGTGGTCTTGTAACACCTGCACGTCATGAACATGCCCCACCCCAGTTCGTAGC
- SerTrpAlaIleLysTrpGluTyrValValLeuLeuPheLeuLeuLeuAlaAspAlaArg
 781 CGTCCTGGGCCATTAAGTGGGAGTACGTCGTTCTCCTGTTCCTTCTGCTTGCAGACGCGC
 GCAGGACCCGGTAATTCACCCTCATGCAGCAAGAGGACAAGGAAGACGAACGTCTGCGCG
- ValCysSerCysLeuTrpMetMetLeuLeuIleSerGlnAlaGluAlaAlaLeuGluAsn 841 GCGTCTGCTCCTGCTTGTGGATGATGCTACTCATATCCCAAGCGGAGGCGGCTTTGGAGA CGCAGACGAGGACGAACACCTACTACGATGAGTATAGGGTTCGCCTCCGCCGAAACCTCT

	0//05
961	PhePheCysPheAlaTrpTyrLeuLysGlyLysTrpValProGlyAlaValTyrThrPheTGTTCTTCTGCTTTGCATGGTATTTGAAGGGTAAGTGGGTGCCCGGAGCGGTCTACACCTACAAGAAGAAGAACGAAC
1021	TyrGlyMetTrpProLeuLeuLeuLeuLeuleuAlaLeuProGlnArgAlaTyrAlaLeuTCTACGGGATGTGGCCTCCTCCTCCTGCTGCTGGTTGCCCCAGCGGGCGTACGCGCAGAGAGAG
1081	AspThrGluValAlaAlaSerCysGlyGlyValValLeuValGlyLeuMetAlaLeuTh TGGACACGGAGGTGGCCGCGTCGTGTGGCGGTGTTGTTCTCGTCGGGTTGATGGCGCTGA ACCTGTGCCTCCACCGGCGCAGCACACCACCACAACAAGAGCAGCCCAACTACCGCGACT
1141	LeuSerProTyrTyrLysArgTyrIleSerTrpCysLeuTrpTrpLeuGlnTyrPheLeu CTCTGTCACCATATTACAAGCGCTATATCAGCTGGTGCTTGTGGTGGCTTCAGTATTTTC GAGACAGTGGTATAATGTTCGCGATATAGTCGACCACGAACACCACCGAAGTCATAAAAG
1201	ThrargValGluAlaGlnLeuHisValTrplleProProLeuAsnValArgGlyGlyArg TGACCAGAGTGGAAGCGCAACTGCACGTGTGGATTCCCCCCCTCAACGTCCGAGGGGGG ACTGGTCTCACCTTCGCGTTGACGTGCACACCTAAGGGGGGGAGTTGCAGGCTCCCCCCG
1261	AspAlaValIleLeuLeuMetCysAlaValHisProThrLeuValPheAspIleThrLys GCGACGCCGTCATCTTACTCATGTGTGCTGTACACCCGACTCTGGTATTTGACATCACCA CGCTGCGGCAGTAGAATGAGTACACACGACATGTGGGCTGAGACCATAAACTGTAGTGGT
1321	LeuLeuleuAlaValPheGlyProLeuTrplleLeuGlnAlaSerLeuLeuLysValProAATTGCTGGCCGTCTTCGGACCCCTTTGGATTCTTCAAGCCAGTTTGCTTAAAGTACTTAAACGACGACCGGCAGAAGCCTGGGGAAACCTAAGAAGTTCGGTCAAACGAATTTCATG
1381	TyrPheValArgValGlnGlyLeuLeuArgPheCysAlaLeuAlaArgLysMetIleGlyCCTACTTTGTGCGCGTCCAAGGCCTTCTCCGGTTCTGCGCGTTAGCGCGGAAGATGATCGGGGTTGAAAACACGCGCAGGTTCCGGAAGAGGCCCAAGACGCGCAATCGCGCCTTCTACTAGC
1441	GlyHisTyrValGlnMetValIleIleLysLeuGlyAlaLeuThrGlyThrTyrValTyrGAGGCCATTACGTGCAAATGGTCATCATTAAGTTAGGGGCGCTTACTGGCACCTATGTTTCTCCCGGTAATGCACGTTTACCAGTAGTAATTCAATCCCCGCGAATGACCGTGGATACAAA
1501	AsnHisleuThrProLeuArgAspTrpAlaHisAsnGlyLeuArgAspLeuAlaValAlaATAACCATCTCACTCCTCTTCGGGACTGGGCGCACAACGGCTTGCGAGATCTGGCCGTGGTATTGGTAGAGTGAGGAGAAGCCCTGACCCGCGTGTTGCCGAACGCTCTAGACCGGCACC
1561	ValGluProValValPheSerGlnMetGluThrLysLeuIleThrTrpGlyAlaAspThr CTGTAGAGCCAGTCGTCTTCTCCCAAATGGAGACCAAGCTCATCACGTGGGGGGCAGATA GACATCTCGGTCAGCAGAAGAGGGGTTTACCTCTGGTTCGAGTAGTGCACCCCCCGTCTAT
1621	AlaAlaCysGlyAspIleIleAsnGlyLeuProValSerAlaArgArgGlyArgGluIleCCGCCGCGTGCGGTGACATCAACGGCTTGCCTGTTTCCGCCCGC
1681	LeuLeuGlyProAlaAspGlyMetValSerLysGlyTrpArgLeuLeuAlaProIleTh TACTGCTCGGGCCAGCCGATGGAATGGTCTCCAAGGGGTGGAGGTTGCTGGCGCCCATCA ATGACGAGCCCGGTCGGCTACCTTACCAGAGGTTCCCCACCTCCAACGACCGCGGGTAGT
1741	AlatyralaginginthrargGlyLeuLeuGlyCysileilethrSerLeuThrGlyArg CGGCGTACGCCCAGCAGACAAGGGGCCTCCTAGGGTGCATAATCACCAGCCTAACTGGCC GCCGCATGCGGGTCGTCTGTTCCCCGGAGGATCCCACGTATTAGTGGTCGGATTGACCGG
1801	AspLysAsnGlnValGluGlyGluValGlnIleValSerThrAlaAlaGlnThrPheLev GGGACAAAAACCAAGTGGAGGGTGAGGTCCAGATTGTGTCAACTGCTGCCCAAACCTTCC CCCTGTTTTTGGTTCACCTCCCACTCCAGGTCTAACACAGTTGACGACGGGTTTGGAAGG
1861	AlaThrCyslleAsnGlyValCysTrpThrValTyrHisGlyAlaGlyThrArgThrIlcTGGCAACGTGCATCAATGGGGTGTGCTGGACTGTCTACCACGGGGCCGGAACGAGGACCAACGTTGCACGTAGTTACCCCACACGACCTGACAGATGGTGCCCCGGCCTTGCTCCTGGT
	AlaserProLysGlyProVallleGlnMetTyrThrAsnValAspGlnAspLeuValGl

FIG. 47-2

1921	TCGCGTCACCCAAGGGTCCTGTCATCCAGATGTATACCAATGTAGACCAAGACCTTGTGG AGCGCAGTGGGTTCCCAGGACAGTAGGTCTACATATGGTTACATCTGGTTCTGGAACACC
1981	TrpProAlaProGlnGlySerArgSerLeuThrProCysThrCysGlySerSerAspLeu GCTGGCCGCTCCGCAAGGTAGCCGCTCATTGACACCCTGCACTTGCGGCTCCTCGGACC CGACCGGGCGAGGCGTTCCATCGGCGAGTAACTGTGGGACGTGAACGCCGAGGAGCCTGG
2041	TyrLeuValThrArgHisAlaAspVallleProValArgArgArgGlyAspSerArgGly TTTACCTGGTCACGAGGCACGCCGATGTCATTCCCGTGCGCCGGCGGGGTGATAGCAGGG AAATGGACCAGTGCTCCGTGCGGCTACAGTAAGGGCACGCGCCCCCCCACTATCGTCCC
2101	SerleuleuSerProArgProIleSerTyrLeuLysGlySerSerGlyGlyProLeuLeu GCAGCCTGCTGTCGCCCCGGCCCATTTCCTACTTGAAAGGCTCCTCGGGGGGTCCGCTGT CGTCGGACGACAGCGGGCCCGGGTAAAGGATGAACTTTCCGAGGAGCCCCCCAGGCGACA
2161	CysProAlaGlyHisAlaValGlyIlePheArgAlaAlaValCysThrArgGlyValAla TGTGCCCCGCGGGGCACGCCGTGGGCATATTTAGGGCCGCGTGTGCACCCGTGGAGTGG ACACGGGGCGCCCCGTGCGGCACCCGTATAAATCCCGGCGCCACACGTGGGCACCTCACC
2221	LysAlaValAspPheIleProValGluAsnLeuGluThrThrMetArgSerProValPhe CTAAGGCGGTGGACTTTATCCCTGTGGAGAACCTAGAGACCATGAGGTCCCCGGTGT GATTCCGCCACCTGAAATAGGGACACCTCTTGGATCTCTGTTGGTACTCCAGGGGCCACA
2281	ThrAspAsnSerSerProProValValProGlnSerPheGlnValAlaHisLeuHisAla TCACGGATAACTCCTCTCCACCAGTAGTGCCCCAGAGCTTCCAGGTGGCTCACCTCCATG AGTGCCTATTGAGGAGAGGTGGTCATCACGGGGTCTCGAAGGTCCACCGAGTGGAGGTAC
2341	ProThrGlySerGlyLysSerThrLysValProAlaAlaTyrAlaAlaGlnGlyTyrLys CTCCCACAGGCAGCGGCAAAAGCACCAAGGTCCCGGCTGCATATGCAGCTCAGGGCTATA GAGGGTGTCCGTCGCCGTTTTCGTGGTTCCAGGGCCGACGTATACGTCGAGTCCCGATAT
2401	ValleuValleuAsnProSerValAlaAlaThrLeuGlyPheGlyAlaTyrMetSerLys AGGTGCTAGTACTCAACCCCTCTGTTGCTGCAACACTGGGCTTTGGTGCTTACATGTCCA TCCACGATCATGAGTTGGGGGAGACAACGACGTTGTGACCCGAAACCACGAATGTACAGGT
2461	AlaHisGlyIleAspProAsnIleArgThrGlyValArgThrIleThrThrGlySerPro AGGCTCATGGGATCGATCCTAACATCAGGACCGGGGTGAGAACAATTACCACTGGCAGCC TCCGAGTACCCTAGCTAGGATTGTAGTCCTGGCCCCACTCTTGTTAATGGTGACCGTCGG
2521	IleThrTyrSerThrTyrGlyLysPheLeuAlaAspGlyGlyCysSerGlyGlyAlaTyr CCATCACGTACTCCACCTACGGCAAGTTCCTTGCCGACGGCGGTGCTCGGGGGGCGCTT GGTAGTGCATGAGGTGGATGCCGTTCAAGGAACGGCTGCCGCCACGAGCCCCCCGCGAA
2581	AspilellelleCysAspGluCysHisSerThrAspAlaThrSerlleLeuGlylleGly ATGACATAATAATTTGTGACGAGTGCCACTCCACGGATGCCACATCCATC
2641	ThrValLeuAspGlnAlaGluThrAlaGlyAlaArgLeuValValLeuAlaThrAlaThr GCACTGTCCTTGACCAAGCAGAGACTGCGGGGGGGGGAGACTGGTTGTGCTCGCCACCGCCA CGTGACAGGAACTGGTTCGTCTCTGACGCCCCCGCTCTGACCAACACGAGCGGTGGCGGT
2701	ProProGlySerValThrValProHisProAsnIleGluGluValAlaLeuSerThrThr CCCCTCCGGGCTCCGTCACTGTGCCCCATCCCAACATCGAGGAGGTTGCTCTGTCCACCA GGGGAGGCCCGAGGCAGTGACACGGGGTAGGGTTGTAGCTCCTCCAACGAGACAGGTGGT
2761	GlyGluileProPheTyrGlyLysAlaIleProLeuGluVallleLysGlyGlyArgHis CCGGAGAGATCCCTTTTTACGGCAAGGCTATCCCCCTCGAAGTAATCAAGGGGGGGAGAC GGCCTCTCTAGGGAAAAATGCCGTTCCGATAGGGGGAGCTTCATTAGTTCCCCCCCTCTG
2821	LeullePheCysHisSerLysLysCysAspGluLeuAlaAlaLysLeuValAlaLeu ATCTCATCTTCTGTCATTCAAAGAAGAAGTGCGACGAACTCGCCGCAAAGCTGGTCGCAT TAGAGTAGAAGACAGTAAGTTTCTTCTTCACGCTGCTTGAGCGGCGTTTCGACCAGCGTA
2881	ACCCGTAGTTACGGCACCGGATGATGGCGCCAGAACTGCACAGGCAGTAGGGCTGGTCGC
	FIC A7 3

FIG. 47-3 SUBSTITUTE SHEET

	59/63 AspValValValAlaThrAspAlaLeuMetThrGlyTyrThrGlyAspPheAspSer
2941	GCGATGTTGTCGTCGTGGCAACCGGATGCCCTCATGACCGGCTATATGGCCGCTGAAGCTGA CGCTACAACAGCAGCACCGTTGGCTACGGGAGTACTGGCCGATATGGCCGCTGAAGCTGA
3001	ValileAspCysAsnThrCysValThrGlnThrValAspPheSerLeuAspProThrPheCGGTGATAGACTGCAATACGTGTGTCACCCAGACAGTCGATTTCAGCCTTGACCCTACCTGCCACTATCTGACGTTATGCACACAGTGGGTCTGTCAGCTAAAGTCGGAACTGGGATGGA
3061	ThrileGluThrileThrLeuProGlnAspAlaValSerArgThrGlnArgArgGlyArg TCACCATTGAGACAATCACGCTCCCCCAGGATGCTGTCTCCCGCACTCAACGTCGGGGCA AGTGGTAACTCTGTTAGTGCGAGGGGGTCCTACGACAGAGGGCGTGAGTTGCAGCCCCGT
3121	ThrGlyArgGlyLysProGlyIleTyrArgPheValAlaProGlyGluArgProSerGlyGACTGGCAGGGGAAGCCAGGCATCTACAGATTTGTGGCACCGGGGAAGCCCCTCCGCCTGCACCGTCCCCTTCGGTCCGTAGATGTCTAAACACCGTGGCCCCCTCGCGGGGAAGCC
3181	MetPheAspSerSerValLeuCysGluCysTyrAspAlaGlyCysAlaTrpTyrGluLeu GCATGTTCGACTCGTCCTCTGTGAGTGCTATGACGCAGGCTGTGCTTGGTATGAGC CGTACAAGCTGAGCAGGCAGGAGACACTCACGATACTGCGTCCGACACGAACCATACTCG
3241	ThrProAlaGluThrThrValArgLeuArgAlaTyrMetAsnThrProGlyLeuProVal TCACGCCCGCGGACTACAGTTAGGCTACGAGCGTACATGAACACCCCGGGGCTTCCCG AGTGCGGGCGGCTCTGATGTCAATCCGATGCTCGCATGTACTTGTGGGGCCCCGAAGGGC
3301	CysGlnAspHisLeuGluPheTrpGluGlyValPheThrGlyLeuThrHisIleAspAla TGTGCCAGGACCATCTTGAATTTTGGGAGGGCGTCTTTACAGGCCTCACTCA
3361	HisPheLeuSerGlnThrLysGlnSerGlyGluAsnLeuProTyrLeuValAlaTyrGlnCCCACTTTCTATCCCAGACAAAGCAGAGTGGGGAGAACCTTCCTT
3421	AlaThrValCysAlaArgAlaGlnAlaProProProSerTrpAspGlnMetTrpLysCys AAGCCACCGTGTGCGCTAGGGCTCAAGCCCCTCCCCCATCGTGGAACCAGATGTGGAAGT TTCGGTGGCACACGCGATCCCGAGTTCGGGGAGGGGGTAGCACCCTGGTCTACACCTTCA
3481	LeuileArgLeuLysProthrLeuHisGlyProthrProLeuLeuTyrArgLeuGlyAla GTTTGATTCGCCTCAAGCCCACCCTCCATGGGCCAACACCCCTGCTATACAGACTGGGCG CAAACTAAGCGGAGTTCGGGTGGGAGGTACCCGGTTGTGGGGACGATATGTCTGACCCGC
3541	ValGlnAsnGluIleThrLeuThrHisProValThrLysTyrIleMetThrCysMetSer CTGTTCAGAATGAAATCACCCTGACGCACCCAGTCACCAAATACATCATGACATGCATG
3601	AlaAspLeuGluValValThrSerThrTrpValLeuValGlyGlyValLeuAlaAlaLeu CGGCCGACCTGGAGGTCGTCACGAGCACCTGGGTGCTCGTTGGCGGCGTCCTGGCTGCTT GCCGGCTGGACCTCCAGCAGTGCTCGTGGACCCACGAGCAACCGCCGCAGGACCGACGAA
3661	AlaAlaTyrCysLeuSerThrGlyCysValValIleValGlyArgValValLeuSerGly TGGCCGCGTATTGCCTGTCAACAGGCTGCGTGGTCATAGTGGGCAGGGTCGTCTTGTCCG

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LysalaLeuGlyLeuGlnThrAlaSerArgGlnAlaGluVallleAlaProAlaVal
3841 AGAAGGCCCTCGGCCTCCTGCAGACCGCGTCCCGTCAGGCAGAGGTTATCGCCCCTGCTG TCTTCCGGGAGCCGGAGGACGTCTGGCGCAGGCAGTCCGTCTCCAATAGCGGGGACGAC ${\tt GlnThrAsnTrpGlnLysLeuGluThrPheTrpAlaLysHisMetTrpAsnPheIleSer}$ FIG. 47-4

ACCGGCGCATAACGGACAGTTGTCCGACGCACCAGTATCACCCGTCCCAGCAGAACAGGC

3721 GGAAGCCGGCAATCATACCTGACAGGGAAGTCCTCTACCGAGAGTTCGATGAGATGGAAG CCTTCGGCCGTTAGTATGGACTGTCCCTTCAGGAGATGGCTCTCAAGCTACTCTACCTTC

CysSerGlnHisLeuProTyrIleGluGlnGlyMetMetLeuAlaGluGlnPheLysGln
3781 AGTGCTCTCAGCACTTACCGTACATCGAGCAAGGGATGATGCTCGCCGAGCAGTTCAAGC
TCACGAGAGTCGTGAATGGCATGTAGCTCGTTCCCTACTACGAGCGGCTCGTCAAGTTCG

 ${\tt LysProAlaIleIleProAspArgGluValLeuTyrArgGluPheAspGluMetGluGlu}$

	60/63
3901	60/63 TCCAGACCAACTGGCAAAAACTCGAGACCTTCTGGGCGAAGCATATGTGGAACTTCATCA AGGTCTGGT1GACCGTTTTTGAGCTCTGGAAGACCCGCTTCGTATACACCTTGAAGTAGT
3961	GlylleGlnTyrLeuAlaGlyLeuSerThrLeuProGlyAsnProAlaIleAlaSerLeu GTGGGATACAATACTTGGCGGGCTTGTCAACGCTGCCTGGTAACCCCGCCATTGCTTCAT CACCCTATGTTATGAACCGCCCGAACAGTTGCGACGGACCATTGGGGCCGGTAACGAAGTA
4021	MetAlaPheThrAlaAlaValThrSerProLeuThrThrSerGlnThrLeuLeuPheAsn TGATGGCTTTTACAGCTGCTGTCACCAGCCCACTAACCACTAGCCAAACCCTCCTCTTCA ACTACCGAAAATGTCGACGACAGTGGTCGGGTGATTGGTGATCGGTTTGGGAGGAGAAGT
4081	IleLeuGlyGlyTrpValAlaAlaGlnLeuAlaAlaProGlyAlaAlaThrAlaPheVal ACATATTGGGGGGTGGGTGCCCAGCTCGCCGCCCCCGGTGCCGCTACTGCCTTTG TGTATAACCCCCCACCCACCGACGGGTCGAGCGGCGGGGGCCACGGCGATGACGGAAAC
4141	GlyAlaGlyLeuAlaGlyAlaAlaIleGlySerValGlyLeuGlyLysValLeuIleAsp TGGGCGCTGGCTTAGCTGGCGCCGCCATCGGCAGTGTTGGACTGGGGAAGGTCCTCATAG ACCCGCGACCGAATCGACCGCGGCGGTAGCCGTCACAACCTGACCCCTTCCAGGAGTATC
4201	IleLeuAlaGlyTyrGlyAlaGlyValAlaGlyAlaLeuValAlaPheLysIleMetSer ACATCCTTGCAGGGTATGGCGGGGGGGGGGGGGGGGGGG
4261	GlyGluValProSerThrGluAspLeuValAsnLeuLeuProAlaIleLeuSerProGly GCGGTGAGGTCCCCTCCACGGAGGACCTGGTCAATCTACTGCCCGCCATCCTCTCGCCCG CGCCACTCCAGGGGAGGTGCCTCCTGGACCAGTTAGATGACGGGCGGTAGGAGAGCGGGC
4321	AlaLeuValValGlyValValCysAlaAlaIleLeuArgArgHisValGlyProGlyGluGAGCCCTCGTAGTCGGCGTGGTCTGTGCAGCAATACTGCGCCGGCACGTTGGCCCGGGCGCCGCCCTCGGGAGCATCAGCCGCACCAGACACGTCGTTATGACGCGGCCGTGCAACCGGGCCCGC
4381	GlyAlaValGlnTrpMetAsnArgLeuIleAlaPheAlaSerArgGlyAsnHisValSer AGGGGGCAGTGCAGTGGATGAACCGGCTGATAGCCTTCGCCTCCCGGGGGAACCATGTTT TCCCCCGTCACGTCA
4441	ProThrHisTyrValProGluSerAspAlaAlaAlaArgValThrAlaIleLeuSerSer CCCCCACGCACTACGTGCCGGAGAGCGATGCAGCTGCCCGCGTCACTGCCATACTCAGCA GGGGGTGCGTGATGCACGGCCTCTCGCTACGTCGACGGGCGCAGTGACGGTATGAGGTCGT
4501	LeuThrValThrGlnLeuLeuArgArgLeuHisGlnTrpIleSerSerGluCysThrThr GCCTCACTGTAACCCAGCTCCTGAGGCGACTGCACCAGTGGATAAGCTCGGAGTGTACCA CGGAGTGACATTGGGTCGAGGACTCCGCTGACGTGGTCACCTATTCGAGCCTCACATGGT
4561	ProCysSerGlySerTrpLeuArgAspIleTrpAspTrpIleCysGluValLeuSerAspCTCCATGCTCCGGTTCCTGGCTAAGGGACATCTGGGACTGGATATGCGAGGTGTTGAGCGGAGGTACGAGGACCGAAGGACCGATTCCCTGTAGACCCTGACCTATACGCTCCACAACTCGC
4621	PheLysThrTrpLeuLysAlaLysLeuMetProGlnLeuProGlyIleProPheValSer ACTTTAAGACCTGGCTAAAAGCTAAGCTCATGCCACGCTGCCTGGGATCCCCTTTGTGT TGAAATTCTGGACCGATTTTCGATTCGA
4681	CysGlnArgGlyTyrLysGlyValTrpArgValAspGlyIleMetHisThrArgCysHis CCTGCCAGCGCGGGTATAAGGGGGTCTGGCGAGTGGACGGCATCATGCACACTCGCTGCC GGACGGTCGCGCCCATATTCCCCCAGACCGCTCACCTGCCGTAGTACGTGTGAGCGACGG
4741	CysGlyAlaGluIleThrGlyHisValLysAsnGlyThrMetArgIleValGlyProArg ACTGTGGAGCTGAGATCACTGGACATGTCAAAAACGGACGATGAGGATCGTCGGTCCTA TGACACCTCGACTCTAGTGACCTGTACAGTTTTTGCCCTGCTACTCCTAGCAGCCAGGAT
4801	ThrCysArgAsnMetTrpSerGlyThrPheProlleAsnAlaTyrThrThrGlyProCys GGACCTGCAGGAACATGTGGAGTGGGACCTTCCCCATTAATGCCTACACCACGGGCCCCT CCTGGACGTCCTTGTACACCTCACCCTGGAAGGGGTAATTACGGATGTGGTGCCCGGGGA
4861	ThrProLeuProAlaProAsnTyrThrPheAlaLeuTrpArgValSerAlaGluGluTyr GTACCCCCTTCCTGCGCCGAACTACACGTTCGCGCTATGGAGGGTGTCTGCAGAGGAAT CATGGGGGGAAGGACGCGGCTTGATGTGCAAGCGCGATACCTCCCACAGACGTCTCCTTA
	FIG. 47-5

	61/63
4921	ValGluIleArgGlnValGlyAspPheHisTyrValThrGlyMetThrThrAspAsnLeu ATGTGGAGATAAGGCAGGTGGGGGACTTCCACTACGTGACGGTATGACTACTGACAATC TACACCTCTATTCCGTCCACCCCCTGAAGGTGATGCACTGCCCATACTGATGACTGTTAG
4981	LysCysProCysGlnValProSerProGluPhePheThrGluLeuAspGlyValArgLeu TCAAATGCCCGTGCCAGGTCCCATCGCCCGAATTTTTCACAGAATTGGACGGGGTGCGCC AGTTTACGGGCACGGTCCAGGGTAGCGGGCTTAAAAAGTGTCTTAACCTGCCCCACGCGG
5041	HisArgPheAlaProProCysLysProLeuLeuArgGluGluValSerPheArgValGlyTACATAGGTTTGCGCCCCCCTGCAAGCCCTTGCTGCGGAAGGGTATCATTCAGAGTAGATGTATCCAAACGCGGGGGGACGTTCGGGAACGACGCCCTCCTCCATAGTAAGTCTCATC
5101	LeuHisGlutyrProValGlySerGlnLeuProCysGluProGluProAspValAlaVal GACTCCACGAATACCCGGTAGGGTCGCAATTACCTTGCGAGCCCGAACCGGACGTGGCCG CTGAGGTGCTTATGGGCCATCCCAGCGTTAATGGAACGCTCGGGCTTGGCCTGCACCGGC
5161	LeuThrSerMetLeuThrAspProSerHislleThrAlaGluAlaGlyArgArgLeu TGTTGACGTCCATGCTCACTGATCCCTCCCATATAACAGCAGAGGCGGCCGGGCGAAGGT ACAACTGCAGGTACGAGTGACTAGGGAGGGTATATTGTCGTCTCCGCCGGCCCGCTTCCA
5221	AlaArgGlySerProProSerValAlaSerSerSerAlaSerGlnLeuSerAlaProSer TGGCGAGGGGATCACCCCCCTCTGTGGCCAGCTCCTCGGCTAGCCAGCTATCCGCTCCAT ACCGCTCCCCTAGTGGGGGGAGACACCCGGTCGAGGAGCCGATCGGTCGATAGGCGAGGTA
5281	LeuLysAlaThrCysThrAlaAsnHisAspSerProAspAlaGluLeuIleGluAlaAsn CTCTCAAGGCAACTTGCACCGCTAACCATGACTCCCCTGATGCTGAGCTCATAGAGGCCA GAGAGTTCCGTTGAACGTGGCGATTGGTACTGAGGGGACTACGACTCGAGTATCTCCGGT
5341	LeuLeuTrpArgGlnGluMetGlyGlyAsnIleThrArgValGluSerGluAsnLysVal ACCTCCTATGGAGGCAGGAGATGGGCGGCAACATCACCAGGGTTGAGTCAGAAAACAAAG TGGAGGATACCTCCGTCCTCTACCCGCCGTTGTAGTGGTCCCAACTCAGTCTTTTGTTTC
5401	ValileLeuAspSerPheAspProLeuValAlaGluGluAspGluArgGluIleSerVal TGGTGATTCTGGACTCCTTCGATCCGCTTGTGGCGGAGGAGGACGACGGGGAGATCTCCG ACCACTAAGACCTGAGGAAGCTAGGCGAACACCGCCTCCTCCTGCTCGCCCTCTAGAGGC
5461	ProAlaGluIleLeuArgLysSerArgArgPheAlaGlnAlaLeuProValTrpAlaArg TACCCGCAGAAATCCTGCGGAAGTCTCGGAGATTCGCCCAGGCCCTGCCCGTTTGGGCGC ATGGGCGTCTTTAGGACGCCTTCAGAGCCTCTAAGCGGGTCCGGGACGGGCAAACCCGCG
5521	ProAspTyrAsnProProLeuValGluThrTrpLysLysProAspTyrGluProProVal GGCCGGACTATAACCCCCCGCTAGTGGAGACGTGGAAAAAGCCCGACTACGAACCACCTG CCGGCCTGATATTGGGGGGCGATCACCTCTGCACCTTTTTCGGGCTGATGCTTGGTGGAC
5581	ValHisGlyCysProLeuProProProLysSerProProValProProProArgLysLys TGGTCCATGGCTGTCCGCTTCCACCTCCAAAGTCCCCTCCTGTGCCTCCGCCTCGGAAGA ACCAGGTACCGACAGGCGAAGGTGGAGGTTTCAGGGGAGGACACGGAGGCGGAGCCTTCT
5641	ArgThrValValLeuThrGluSerThrLeuSerThrAlaLeuAlaGluLeuAlaThrArg AGCGGACGGTGGTCCTCACTGAATCAACCCTATCTACTGCCTTGGCCGAGCTCGCCACCA TCGCCTGCCACCAGGAGTGACTTAGTTGGGATAGATGACGGAACCGGCTCGAGCGGTGGT
5701	SerPheGlySerSerSerThrSerGlyIleThrGlyAspAsnThrThrThrSerSerGlu GAAGCTTTGGCAGCTCCTCAACTTCCGGCATTACGGGCGACAATACGACAACATCCTCTG CTTCGAAACCGTCGAGGAGTTGAAGGCCGTAATGCCCGCTGTTATGCTGTTGTAGGAGAC
5761	ProAlaProSerGlyCysProProAspSerAspAlaGluSerTyrSerSerMetProPro AGCCCGCCCTTCTGGCTGCCCCCCGACTCCGACGCTGAGTCCTATTCCTCCATGCCCC TCGGGCGGGAAGACCGACGGGGGGGCTGAGGCTGCGACTCAGGATAAGGAGGTACGGGG
5821	LeuGluGlyGluProGlyAspProAspLeuSerAspGlySerTrpSerThrValSerSer CCCTGGAGGGGAGCCTGGGGATCCGGATCTTAGCGACGGTCATGGTCAACGGTCAGTA GGGACCTCCCCCTCGGACCCCTAGGCCTAGAATCGCTGCCCAGTACCAGTTGCCAGTCAT
•	GluAlaAsnAlaGluAspValValCysCysSerMetSerTyrSerTrpThrGlyAlaLeu
	FIG. 47-6

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	62/63
5881	GTGAGGCCAACGCGGAGGATGTCGTGTGCTGCTCAATGTCTTACTCTTGGACAGGCGCAC. CACTCCGGTTGCGCCTCCTACAGCACACGACGAGTTACAGAATGAGAACCTGTCCGCGTG
5941	ValThrProCysAlaAlaGluGluGlnLysLeuProIleAsnAlaLeuSerAsnSerLeu TCGTCACCCCGTGCGCCGCGGAAGAACAGAAACTGCCCATCAATGCACTAAGCAACTCGT AGCAGTGGGGCACGCGGCGCCTTCTTGTCTTTGACGGGTAGTTACGTGATTCGTTGAGCA
6001	LeuArgHisHisAsnLeuValTyrSerThrThrSerArgSerAlaCysGlnArgGlnLys TGCTACGTCACCACATTTGGTGTATTCCACCACCTCACGCAGTGCTTGCCAAAGGCAGA ACGATGCAGTGGTGTTAAACCACATAAGGTGGTGGAGTGCGTCACGAACGGTTTCCGTCT
6061	LysValThrPheAspArgLeuGlnValLeuAspSerHisTyrGlnAspValLeuLysGlu AGAAAGTCACATTTGACAGACTGCAAGTTCTGGACAGCCATTACCAGGACGTACTCAAGG TCTTTCAGTGTAAACTGTCTGACGTTCAAGACCTGTCGGTAATGGTCCTGCATGAGTTCC
6121	VallysAlaAlaSerLysVallysAlaAsnLeuLeuSerValGluGluAlaCysSer AGGTTAAAGCAGCGGCGTCAAAAGTGAAGGCTAACTTGCTATCCGTAGAGGAAGCTTGCA TCCAATTTCGTCGCCGCAGTTTTCACTTCCGATTGAACGATAGGCATCTCCTTCGAACGT
6181	LeuThrProProHisSerAlaLysSerLysPheGlyTyrGlyAlaLysAspValArgCys GCCTGACGCCCCACACTCAGCCAAATCCAAGTTTGGTTATGGGGCAAAAGACGTCCGTT CGGACTGCGGGGGTGTGAGTCGGTTTAGGTTCAAACCAATACCCCGTTTTCTGCAGGCAA
6241	HisAlaArgLysAlaValThrHisIleAsnSerValTrpLysAspLeuLeuGluAspAsn GCCATGCCAGAAAGGCCGTAACCCACATCAACTCCGTGTGGAAAGACCTTCTGGAAGACA CGGTACGGTCTTTCCGGCATTGGGTGTAGTTGAGGCACACCTTTCTGGAAGACCTTCTGT
6301	ValThrProlleAspThrThrlleMetAlaLysAsnGluValPheCysValGlnProGlu ATGTAACACCAATAGACACTACCATCATGGCTAAGAACGAGGTTTTCTGCGTTCAGCCTG TACATTGTGGTTATCTGTGATGGTAGTACCGATTCTTGCTCCAAAAGACGCAAGTCGGAC
6361	LysGlyGlyArgLysProAlaArgLeuIleValPheProAspLeuGlyValArgValCys AGAAGGGGGGTCGTAAGCCAGCTCGTCTCATCGTGTTCCCCGATCTGGGCGTGCGCGTGT TCTTCCCCCCAGCATTCGGTCGAGCAGAGTAGCACAAGGGGCTAGACCCGCACGCGCACA
6421	GluLysMetAlaLeuTyrAspValValThrLysLeuProLeuAlaValMetGlySerSer GCGAAAAGATGGCTTTGTACGACGTGGTTACAAAGCTCCCCTTGGCCGTGATGGGAAGCT CGCTTTTCTACCGAAACATGCTGCACCAATGTTTCGAGGGGAACCGGCACTACCCTTCGA
6481	TyrGlyPheGlnTyrSerProGlyGlnArgValGluPheLeuValGlnAlaTrpLysSer CCTACGGATTCCAATACTCACCAGGACAGCGGGTTGAATTCCTCGTGCAAGCGTGGAAGT GGATGCCTAAGGTTATGAGTGGTCCTGTCGCCCAACTTAAGGAGCACGTTCGCACCTTCA
6541	LysLysThrProMetGlyPheSerTyrAspThrArgCysPheAspSerThrValThrGlu CCAAGAAAACCCCAATGGGGTTCTCGTATGATACCCGCTGCTTTGACTCCACAGTCACTG GGTTCTTTTGGGGTTACCCCAAGAGCATACTATGGGCGACGAAACTGAGGTGTCAGTGAC
6601	SerAspIleArgThrGluGluAlaIleTyrGlnCysCysAspLeuAspProGlnAlaArg AGAGCGACATCCGTACGGAGGAGGCAATCTACCAATGTTGTGACCTCGACCCCCAAGCCC TCTCGCTGTAGGCATGCCTCCTCCGTTAGATGGTTACAACACTGGAGCTGGGGGTTCGGG
6661	ValalaileLysSerLeuThrGluArgLeuTyrValGlyGlyProLeuThrAsnSerArg GCGTGGCCATCAAGTCCCTCACCGAGAGGCTTTATGTTGGGGGCCCTCTTACCAATTCAA CGCACCGGTAGTTCAGGGAGTGGCTCTCCGAAATACAACCCCCGGGAGAATGGTTAAGTT
6721	GlyGluAsnCysGlyTyrArgArgCysArgAlaSerGlyValLeuThrThrSerCysGly GGGGGGAGAACTGCGGCTATCGCAGGTGCCGCGCGGGGGGGG
6781	CATTGTGGGAGTGAACGATGTAGTTCCGGGCCCGTCGGACAGCTCGGCGTCCCGAGGTCC
6841	CysThrMetLeuValCysGlyAspAspLeuValVallleCysGluSerAlaGlyValGlr ACTGCACCATGCTCGTGTGTGGCGACGACTTAGTCGTTATCTGTGAAAGCGCGGGGGTCC TGACGTGGTACGAGCACACCGCTGCTGAATCAGCAATAGACACTTTCGCGCCCCCAGG

6901	GluAspAlaAlaSerLeuArgAlaPheThrGluAlaMetThrArgTyrSerAlaProPro AGGAGGACGCGGCGAGCCTGAGAGCCTTCACGGAGGCTATGACCAGGTACTCCGCCCCCC TCCTCCTGCGCCGCCTCGGACTCTCGGAAGTGCCTCCGATACTGGTCCATGAGGCGGGGG
6961	GlyAspProProGlnProGluTyrAspLeuGluLeuIleThrSerCysSerSerAsnVal CTGGGGACCCCCACAACCAGAATACGACTTGGAGCTCATAACATCATGCTCCTCCAACG GACCCCTGGGGGGTGTTGGTCTTATGCTGAACCTCGAGTATTGTAGTACGAGGAGGTTGC
7021	SerValAlaHisAspGlyAlaGlyLysArgValTyrTyrLeuThrArgAspProThrThr TGTCAGTCGCCCACGACGCGCTGGAAAGAGGGTCTACTACCTCACCCGTGACCCTACAA ACAGTCAGCGGGTGCTGCCGCGACCTTTCTCCCAGATGATGGAGTGGGCACTGGGATGTT
7081	ProLeuAlaArgAlaAlaTrpGluThrAlaArgHisThrProValAsnSerTrpLeuGly CCCCCCTCGCGAGAGCTGCGTGGGAGACAGCAGACACTCCAGTCAATTCCTGGCTAG GGGGGGAGCGCTCTCGACGCACCCTCTGTCGTTCTGTGTGAGGTCAGTTAAGGACCGATC
7141	AsnilelleMetPheAlaProThrLeuTrpAlaArgMetIleLeuMetThrHisPhePheGCAACATAATCATGTTTGCCCCCACACTGTGGGCGAGGATGATACTGATGACCCATTTCTCGTTGTTGTATTAGTACAAACGGGGGTGTGACACCCGCTCCTACTATGACTACTGGGTAAAGA
7201	SerValleuIleAlaArgAspGlnLeuGluGlnAlaLeuAspCysGluIleTyrGlyAla TTAGCGTCCTTATAGCCAGGGACCAGCTTGAACAGGCCCTCGATTGCGAGATCTACGGGG AATCGCAGGAATATCGGTCCCTGGTCGAACTTGTCCGGGAGCTAACGCTCTAGATGCCCC
7251	CystyrSerileGluProLeuAspLeuProProllelleGlnArgLeu CCTGCTACTCCATAGAACCACTTGATCTACCTCCAATCATTCAAAGACTC GGACGATGAGGTATCTTGGTGAACTAGATGGAGGTTAGTAAGTTTCTGAG

FIG. 47-8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/04125

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all). 6 According to International Patent Classification (IPC) or to both National Classification and IPC INT. Cl. (4) A61K 39/12; C12Q 1/70; C12N 15/00; C07K 7/10; A61K 37/04; A61K 37/ 02; C07H 15/12 US Cl. 424/89; 435/5; 435/6; 435/172-2; 530/324; 530/350; 530/387; 536/27 :530/324:530/350:530/387:536/27; II. FIELDS SEARCHED Minimum Documentation Searched 7 Classification Symbols Classification System 424/89; 435/5; 435/6; 435/172.2; 530/324; 530/350; 530/387; 536/27; U.S. Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched Online search conducted on chemical Abstracts and Biosis. III. DOCUMENTS CONSIDERED TO BE RELEVANT Relevant to Claim No. 13 Citation of Document, 11 with Indication, where appropriate, of the relevant passages 12 1-17,20-21 Journal of Virology, Volume 62 No.2 25,27-28 issued February 1988, Weiner et al ŖΧ 40 "A single antigenomic open frame of the Hepatitis Delta virus encodes the epitope(s) of both Hepatitis Delta antigen polypeptides p 24 Delta and p 27 Delta". See pages 594-599. Nature, Volume 323, issued 9 October 1986, Wang et al, "Structure, sequence and expression of the Hepatitis Delta 1-17, 20-21 X 25,27 28,40 viral genome". See pages 508-513 Journal of Virological Methods, Volum 8 22-24 1984, Neurath et al "Strategies for 29-31 Α detection of transfusion-transmitted viruses eluding identification by conventional serologic tests. II. Dectec tion o host DNA in Human Plasmas with elevated alanine aminotransferase" See "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention e Special categoripaga accumulation "A" document defining the general state of the art which is not considered to be of particular relevance earlier document but published on or after the international filing date "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled "O" document referring to an oral disclosure, use, exhibition or document published prior to the international filing date but later than the priority date claimed "4" document member of the same patent family IV. CERTIFICATION Date of Mailing of this International Search Report Date of the Actual Completion of the International Search 0 3 APR 1989 63 February 1939 Signature of Authorized Officer of Sunau ad International Searching Authority Abdel A. Mohamed ISA/US

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II. DOCUM	UMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
ategory •	Citation of Document, with Indication, where appropriate, of the relevant passages	Relevant to Claim No	
Y	U.S.A. 4,673,634, Seto et al, Published 16 June, 1987 (Column 1-10)	9-13,20 22-24, 29-34	
Y	U.S.A 4,702,909, Villarejos et al, Published 27 October, 1987 (column 1-10)	9-13, 18-20,22-24 26,29,34,39	
Y	WO 82/00205, Coursget et al, Published 21 January 1982 "Non-A Non-B Hepatitis Assay and Vaccine" (See the Whole Document)	22-34	
Y	WO 87/05930, Foung et al, Published 8 October 1987 "Immortalized Virus- specific tissue cells" (See the whole Document)	35-37	
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	International Application No.
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PURTHE	A INFORMATION CONTINUED FROM THE SECOND
	SERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE I
This inter	national search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:
ı. 🗌 Clai	m numbers . because they relate to subject matter 12 not required to be searched by this Authority, namely:
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	to the Atomic and American
2. Clai	m numbers, because they relate to parts of the international application that do not comply with the prescribed require- ts to such an extent that no meaningful international search can be carried out ¹³ , specifically:
mer	is to such an extent that no meaningful international Space. Can be desired as the same of
_	m numbers, because they are dependent claims not drafted in accordance with the second and third sentences of
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	FRuie 6.4(a).
	SSERVATIONS WHERE UNITY OF INVENTION IS LACKING?
This Inte	national Searching Authority found multiple inventions in this International application as follows:
	See Attached Sheet
	nee Attached Sheet
	•
1 X7 A-	all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
of t	he international application. To lephone Practice (See Artached Sheet)
2. As	coly some of the required additional search fees were timely paid by the applicant, this international search report covers only
tho	se claims of the international application for which fees were paid, specifically claims:
, C	required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to
No the	required additional search tees were timely paid by the applicant consequently, the internal search tees were timely paid by the applicant consequently, the internal search tees were timely paid by claim numbers:
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4. 🔲 As	all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not te payment of any additional fee.
	n Protest
The	additional search fees were accompanied by applicant's protest.

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ATTACHMENT TO FORM PCT/ISA/210, Part VI. 1

Telephone Approval:

\$280.00 payment is approved by the attorney Gladys H. Monroy on 3 February 1989 for Groups I,II and III; charge to Deposit Account No. 03-1952.

REASONS FOR HOLDING LACK OF UNITY IF INVENTION:

The invention as defined by Group II (claim 32) classified in class 424, subclass 89 and Group III (claim 40) classified in class 435, subclass 172.3, are drawn to a further alternative composition and a further alternative method additional to the compositions and methods of Group I and accordingly, lack of unity of invention under PCT Rule 13.2.

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Attachment to Form PCT/ISA/210, Part VI.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING:

I Claim 1-31 and 33-39, Drawn to purified HCV, polynucleotides thereof, Polypeptide therefore, recombinant vectors, hosts, Antibodies thereto and probes thereto; classified in classes 435,536,530 and 424; subclasses 235,27, 300+ and 89+.

II Claim 32, Drawn to a polypeptide vaccine; classified in class 424, subclass 89.

III Claim 40, drawn to a method of isolating cDNA; classified in class 435, subclass 172.3.